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from
BOYCE THOMPSON
INSTITUTE

13099
R6667



VOLUME 18
1954-1957

Published Quarterly by
BOYCE THOMPSON INSTITUTE FOR PLANT RESEARCH, INC.
Yonkers 3, New York

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ERRATA

Vol. 18, pages 43 and 44, under "EFFECT OF NUTRIENTS" and "TABLE I," "exogenous" refers to total oxygen consumption in presence of sucrose, rather than the difference between this and the endogenous consumption.

Vol. 18, page 244, line 5, "*Verbena hydrida*" should read "*Verbena hybrida*"

Vol. 18, page 258, Figure 4, "Cytochrome c" in descriptive box should read "Cytochrome c+sodium succinate"

Vol. 18, page 357, line 4, author "A. M. MEISS" should read "A. N. MEISS"

Vol. 18, page 406, TABLE VI, column 4, " pK'_b " should read " pK'_b "

Vol. 18, page 455, TABLE I, columns 3 and 4 should read as follows:

| Neutral equivalent | |
|--------------------|-------|
| Calcd. | Found |

CYCLETHRIN, A NEW INSECTICIDE OF THE PYRETHRINS-TYPE¹

HARRY L. HAYNES,² HOWARD R. GUEST,³ HARRY A. STANSBURY,³
ANTHONY A. SOUSA,⁴ AND ANDREW J. BORASH⁴

SUMMARY

A new insecticide, cyclethrin, 3-(2-cyclopentenyl)-2-methyl-4-oxo-2-cyclopentenyl chrysanthemummonocarboxylate, has been synthesized which possesses many of the attributes of natural pyrethrins. Toxicity to mammals is of the same nature as that of allethrin and pyrethrins. Cyclethrin is synergized by the common pyrethrins synergists more readily than allethrin.

Insecticide tests have shown that, with synergists piperonyl butoxide and sulf-oxide, 1.6 to 1.7 times the amount of cyclethrin as pyrethrins is needed to provide excellent Grade AA space sprays for house flies; double the dosages of allethrin and piperonyl butoxide are required to equal the pyrethrins sprays. Tests on house flies with low-pressure aerosols containing synergists with no DDT indicate 1.8 times as much cyclethrin and 3.7 to 5.8 times the amount of allethrin are required to equal pyrethrins. When DDT is added to such mixtures the knockdown and kill of house flies by the allethrin formulations is improved so it equals that of cyclethrin.

Sulfoxide and piperonyl butoxide synergize cyclethrin in oil contact sprays better than allethrin for knockdown of German roaches. Cyclethrin with sulfoxide provides higher kill when compared with similar allethrin mixtures.

Synergized cyclethrin shows considerable promise for control of biting flies attacking dairy cows when used in oil-space and repellent-type sprays, in water-based sprays and in treadle-sprayer concentrates.

Aqueous sprays containing certain synergists activate cyclethrin to a greater degree than allethrin for control of bean aphids, spider mite species, and Mexican bean beetle larvae.

Cyclethrin was found to be equal to allethrin when the insecticides were used with and without synergists in dust mixtures for killing rice weevils on wheat grain.

INTRODUCTION

Pyrethrum extracts have been used in household and livestock sprays for over 30 years because of their quick paralytic effect on insects and relative safety for mammals. By laborious and brilliant effort the various pyrethrins and cinerins were isolated and identified by LaForge and his co-workers. In due course of time the Bureau of Entomology and Plant Quarantine chemists, Schechter, Green, and LaForge (7, 8) were able to synthesize the allyl homologue of cinerin I which was very similar chemi-

¹ This article was preprinted October 1, 1954.

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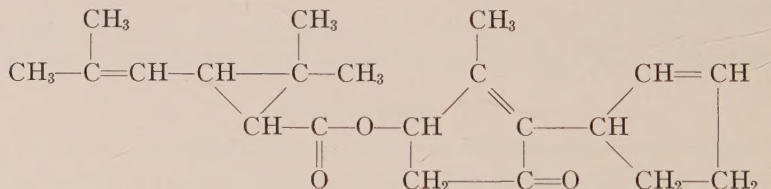
cally to one of the principal ingredients in pyrethrum. This material was given the name of allethrin.

Allethrin has been available commercially since 1950 but has been found to have three limitations that restrict its usefulness. It does not have as quick paralytic effect on flies as natural pyrethrins, it is not synergized effectively by the common pyrethrins synergists, and it is somewhat less effective on agricultural insects.

In view of these limitations of allethrin and the obvious need of the United States for a domestic source of a safe pyrethrins-like insecticide that would free us of dependence upon foreign sources for pyrethrum, a systematic search was begun among the compounds related to allethrin. One of these prepared by substituting a cyclopentenyl group for the allyl chain to give 3-(2-cyclopentenyl)-2-methyl-4-oxo-2-cyclopentenyl chrysanthemummonocarboxylate proved to be of particular interest. This chemical, designated in this paper by the name of cyclethrin, possessed low mammalian toxicity (2), was found to be similar to allethrin and pyrethrins in its activity on house flies, and was synergized more readily than allethrin with the common pyrethrins synergists. The present report is concerned with a brief description of certain insecticidal and chemical aspects of cyclethrin.

MATERIALS AND METHODS

Description of the chemical. Cyclethrin was synthesized by esterification of *dl-cis* and *trans* chrysanthemummonocarboxylic acid with *dl*-2-(2-cyclopentenyl)-3-methyl-2-cyclopenten-4-ol-lone. The product was a mixture of eight possible stereo-isomers of the following molecular formula:



Laboratory samples have been prepared which varied in purity from approximately 80 to 95 per cent as determined by an adaptation of the ethylene diamine method of analyzing allethrin (5) (Hogsett, Kacy, and Johnson advised the authors in a personal communication that the allethrin method gave reliable results provided the ethylene diamine reaction was permitted to proceed for three hours instead of two).

Cyclethrin of a purity of 95 per cent is a viscous, straw-colored liquid with a specific gravity of 1.020 at 20°/20° and a refractive index (n_D^{30}) of 1.5170. Boiling points were not obtained because the material decomposes at elevated temperatures. Molecular distillation was accom-

plished with a spinning-disk type apparatus at a rotor temperature of 100° C. at an indicated pressure of .02 mm. of mercury. The material has been found to be compatible with other insecticides and diluents commonly used in household oil sprays, aerosols and livestock sprays.

Formulation. The cyclethrin was compared to pyrethrins (standard 20 per cent concentrates in petroleum distillate or OTI) and commercial grade allethrin of 75 to 88 per cent purity from Carbide and Carbon Chemicals Co. Oil sprays were prepared by diluting the concentrates in refined petroleum distillate. Low-pressure aerosols were prepared by mixing the 15 per cent nonvolatile portion containing insecticides, petroleum distillate and approximately 5 per cent methylated naphthalene solvents with 85 per cent propellants. The propellants contained equal parts of trichlorofluoromethane and dichlorodifluoromethane. The mixture was prepared in a 12-ounce beer-can type dispenser equipped with a pressure release valve. Aqueous sprays, to be used against crop pests, were prepared by emulsifying the oil concentrates. The emulsifier, Tergitol Dispersant NPX (an alkylphenyl polyethylene glycol ether, Carbide and Carbon Chemicals Co.) was used at the rate of 10 per cent by weight of the insecticide mixture. Acetone to equal 10 per cent of the final volume of the aqueous spray was added as a solvent. Dusts for use on wheat grain were prepared by impregnating the wheat flour diluent with insecticides from acetone slurries.

Test method. Tests on the common house fly, *Musca domestica* L., were made according to the large group Peet-Grady method (6). Low-pressure aerosol tests on house flies were conducted in the Peet-Grady chamber according to the large group Aerosol Test Method for Flying Insects (1).

Spray tests with the male German cockroach, *Blattella germanica* L., were conducted according to the Official Cockroach Spray Method of the Chemical Specialties Manufacturers Association (3).

The apparatus used and conditions under which sprayed plants were kept for the tests on Mexican bean beetle larvae, *Epilachna varivestris* Muls., bean aphid, *Aphis fabae* Scop., and greenhouse red spider mite species, *Tetranychus bimaculatus* Harvey and *T. althaea* McGregor have been described previously (4). For Mexican bean beetle larvae and red spider mites, bean (*Phaseolus vulgaris* L.) plants of the variety "Tendergreen" were sprayed on a turntable at 40 pounds' pressure until the plants were thoroughly wetted and the spray was observed to run off leaves. One hour after spraying, each plant was infested with four third-instar Mexican bean beetle larvae by enclosing the leafy portion of the plant and the larvae in a spherical wire cage. Observations for mortality and amount of feeding were taken after 72 hours.

In the red spider test, each plant was infested with approximately 100 adults 24 hours prior to spraying. Counts of mortality were taken 48

hours after the insecticidal sprays were applied. For the aphid tests, by the spray method, clay pots 2.5 inches in diameter containing 6 to 10 *Nasturtium* plants infested with bean aphids were removed from a stock culture. The number of aphids to be tested was standardized at 100 to 150 adults and nymphs by trimming off plants containing excess aphids. The plants were then sprayed in the same manner as that described for mite testing. After spraying, the potted plants were placed on their sides in a Petri dish on a piece of white standard mimeograph paper which was previously ruled into squares to facilitate counting. The paper was ringed with Tanglefoot glue to prevent the test insects from escaping. Counts of the insects which had fallen to the paper and those that remained on the plants were made 24 hours after spray application. All percentage control or mortality figures were corrected for natural mortality in the untreated lots.

Grain protectant tests were made on wheat (*Triticum* sp.) to be infested with the rice weevil, *Sitophilus oryza* (L.). Two-ounce lots of wheat were treated, at the rate of 50, 100 and 200 pounds per 1000 bushels of wheat, with dusts containing 0.8 to 1.2 per cent of toxicant and 1.1 to 1.6 per cent piperonyl butoxide or sulfoxide in whole-wheat flour diluent. Thirty-day-old weevils were then added to each jar (two replicates per concentration) and records were taken on survival after seven days at room temperature.

Field repellency tests were made with flies on Guernsey and Holstein cattle. The animals in a herd heavily infested with populations of house flies, horn flies [*Siphona irritans* (L.)], or stable flies [*Stomoxys calcitrans* (L.)] were sprayed with a quart of aqueous emulsion or two fluid ounces of oil solution. Animals received approximately 0.5 ml. dosages of oil concentrates two times daily in the treadle-sprayer application. Records were taken on infestations daily for treadle-sprayer applications, over a period of seven days for aqueous sprays and two days for oil solution, to determine severity of infestation.

EXPERIMENTAL RESULTS

Space sprays on house flies. The relative performance of pyrethrins, allethrin and cyclothrin without synergists for knockdown and kill of the common house fly was determined. Large group Peet-Grady results for 24-hour mortality and 3-, 5-, and 10-minute knockdown are shown in Table I.

The log probability curves in Figure 1 represent the average of 32 tests for each material at three concentrations: 50, 100 and 200 mg. in 100 ml. of refined petroleum distillate. Curves are presented for the three materials for 24-hour kill and 10-minute knockdown. These tests were run over a period of two years to take into account variation normally encountered

TABLE I

EFFECTIVENESS OF PYRETHRINS, ALLETHRIN, AND CYCLETHRIN IN SPACE SPRAYS FOR KNOCKDOWN AND KILL OF HOUSE FLIES*

| Insecticide | Dosage** (mg.) | % Kill 24 hrs. | % Knockdown | | |
|-------------|-------------------|-------------------|-------------|--------|---------|
| | | | 3 Min. | 5 Min. | 10 Min. |
| Pyrethrins | 200 | 59 | 94 | 96 | 98 |
| | 100 | 33 | 87 | 91 | 94 |
| | 50 | 19 | 69 | 76 | 81 |
| Allethrin | 200 | 63 | 94 | 96 | 98 |
| | 100 | 35 | 88 | 92 | 95 |
| | 50 | 18 | 71 | 79 | 85 |
| Cyclothrin | 200 | 59 | 91 | 95 | 97 |
| | 100 | 34 | 78 | 87 | 92 |
| | 50 | 22 | 57 | 69 | 78 |
| OTI*** | | 36 | 91 | 93 | 95 |

* Figures for kill and knockdown are averages of 32 tests for pyrethrins, allethrin, and cyclothrin, and 171 tests for the OTI. Tests were conducted over the period from March 1952 to March 1954.

** Mg. of chemical in 100 ml. of refined petroleum distillate.

*** Official Test Insecticide contains 100 mg. of pyrethrins in 100 ml. of refined petroleum distillate.

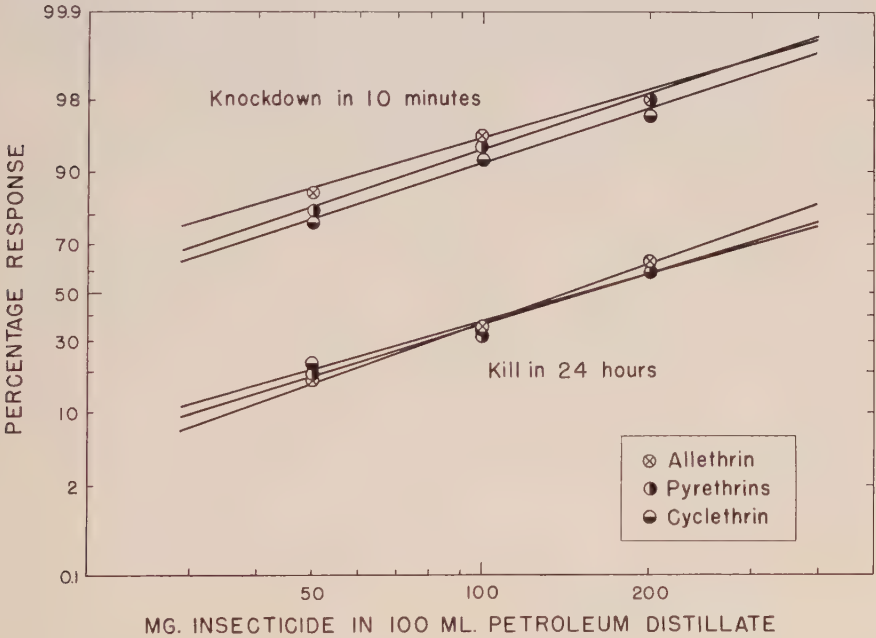


FIGURE 1. Dosage response of house flies to cyclothrin, pyrethrins, and allethrin in large group Peet-Grady tests. (No synergists used.)

in the Peet-Grady fly strain. Statistically there is no difference between materials for kill of the down flies in 24 hours, nor for 10-minute knock-down at dosages of 200, 100 and 50 mg. Pyrethrins and allethrin are slightly more effective at 3 minutes than cyclothrin at all three concentrations (Table I). They are also slightly better for 5-minute knockdown at 100- and 50-mg. dosages.

In the evaluation of an oil spray by the large group Peet-Grady method, it is recognized that flies sprayed with allethrin are not paralyzed so rapidly as those sprayed with pyrethrins. At 3 minutes, allethrin-sprayed flies are buzzing and spinning around on the floor as compared with more nearly total paralysis for pyrethrins-treated flies. Cyclothrin resembles pyrethrins in its paralytic effect. Quicker immobilization or paralysis of down flies is obtained with cyclothrin than with allethrin. Quicker immobilization is more apparent and significant when these materials are evaluated at economical use levels with synergists.

Of greater practical significance is the performance of cyclothrin when compared with pyrethrins or allethrin tested at use levels with synergists. It was determined early in space-spray tests that cyclothrin was synergized for kill in a much greater degree than allethrin. Typical data for mixtures containing synergists, sulfoxide, which is 1,2-methylenedioxy-4-[2-(octylsulfinyl)propyl]benzene, piperonyl butoxide, which is α -[2-(2-*n*-butoxyethoxy)ethoxy]-4,5-methylenedioxy-2-propyltoluene, *n*-propyl isome, which is di-*n*-propyl 6,7-methylenedioxy-3-methyl-1,2,3,4-tetrahydro-

TABLE II

EFFECTIVENESS OF FOUR SYNERGISTS WITH CYCLOTHRIN AND ALLETHRIN IN SPACE SPRAYS FOR KNOCKDOWN AND KILL OF HOUSE FLIES

| Formulation* | | No. of tests | % Kill 24 hrs. | % Knockdown | | | OTI difference | |
|-------------------|----------------------------|--------------|----------------|-------------|--------|---------|----------------|--------------------|
| Insecticide (mg.) | Synergist (mg.) | | | 3 Min. | 5 Min. | 10 Min. | Kill 24 hrs. | Knock-down 10 min. |
| Cyclothrin 42 | Piperonyl butoxide 200 | 8 | 65 | 73 | 82 | 93 | +28 | - 2 |
| Allethrin 42 | Piperonyl butoxide 200 | 8 | 45 | 76 | 83 | 90 | + 8 | - 5 |
| OTI | | 13 | 37 | 90 | 93 | 95 | | |
| Cyclothrin 50 | Sulfoxide 250 | 6 | 75 | 93 | 95 | 97 | +36 | + 2 |
| Allethrin 50 | Sulfoxide 250 | 6 | 37 | 88 | 90 | 91 | 0 | - 4 |
| OTI | | 8 | 37 | 93 | 95 | 98 | | |
| Cyclothrin 42 | <i>n</i> -Propyl isome 400 | 5 | 56 | 69 | 74 | 82 | +16 | -11 |
| Allethrin 42 | <i>n</i> -Propyl isome 400 | 5 | 42 | 66 | 71 | 79 | + 2 | -14 |
| OTI | | 9 | 40 | 89 | 91 | 93 | | |
| Cyclothrin 30 | Compound 6266 1000 | 9 | 47 | 85 | 91 | 95 | +11 | 0 |
| Allethrin 30 | Compound 6266 1000 | 9 | 33 | 86 | 90 | 93 | - 3 | - 2 |
| OTI | | 14 | 36 | 92 | 94 | 95 | | |

* Mg. of chemical in 100 ml. of refined petroleum distillate.

TABLE III

EFFECTIVENESS OF DIFFERENT RATIOS OF SULFOXIDE WITH CYCLETHRIN AND PYRETHRINS IN SPACE SPRAYS FOR KNOCKDOWN AND KILL OF HOUSE FLIES*

| Insecticide** | | Sulfoxide** (mg.) | % Kill 24 hrs. | % Knockdown | | | OTI difference | |
|---------------|------------------------|----------------------|-------------------|-------------|--------|---------|-----------------|----------------------|
| Name | Concentration (mg.) | | | 3 Min. | 5 Min. | 10 Min. | Kill 24 hrs. | Knockdown 10 min. |
| Cyclethrin | 25 | 100 | 39 | 59 | 71 | 84 | - 7 | -9 |
| | 25 | 200 | 52 | 61 | 74 | 86 | + 6 | -7 |
| | 25 | 300 | 63 | 74 | 80 | 91 | +17 | -2 |
| | 25 | 400 | 72 | 69 | 83 | 91 | +26 | -2 |
| | 50 | 100 | 56 | 69 | 84 | 92 | +10 | -1 |
| | 50 | 200 | 71 | 74 | 87 | 94 | +25 | +1 |
| | 50 | 300 | 79 | 79 | 89 | 95 | +33 | +2 |
| | 50 | 400 | 87 | 82 | 91 | 96 | +41 | +3 |
| | 75 | 100 | 70 | 79 | 90 | 95 | +24 | +2 |
| | 75 | 200 | 77 | 81 | 91 | 96 | +31 | +3 |
| | 75 | 300 | 86 | 82 | 91 | 96 | +40 | +3 |
| | 75 | 400 | 93 | 85 | 92 | 97 | +47 | +4 |
| Pyrethrins | 25 | 100 | 45 | 74 | 83 | 91 | - 1 | -2 |
| | 25 | 200 | 61 | 78 | 86 | 93 | +15 | 0 |
| | 25 | 300 | 78 | 83 | 92 | 96 | +32 | +3 |
| | 25 | 400 | 89 | 88 | 94 | 98 | +43 | +5 |
| | 50 | 100 | 65 | 83 | 91 | 95 | +19 | +2 |
| | 50 | 200 | 81 | 91 | 94 | 97 | +35 | +4 |
| | 50 | 300 | 94 | 91 | 96 | 98 | +48 | +5 |
| | 50 | 400 | 95 | 91 | 96 | 98 | +49 | +5 |
| | 75 | 100 | 79 | 89 | 93 | 97 | +33 | +4 |
| | 75 | 200 | 94 | 92 | 95 | 98 | +48 | +5 |
| | 75 | 300 | 95 | 93 | 96 | 98 | +49 | +5 |
| | 75 | 400 | 98 | 94 | 98 | 99 | +52 | +6 |
| OTI | | | 46 | 88 | 91 | 93 | | |

* Figures for synergist and toxicant are averages of 10 paired tests compared with 30 tests for the OTI.

** Mg. of chemical in 100 ml. of refined petroleum distillate.

naphthalene-1,2-dicarboxylate, and Synergist 6266 (an experimental synergist of Carbide and Carbon Chemicals Co.), each with cyclethrin and allethrin are shown in Table II. The tests were not designed to compare the relative efficiencies of the synergists with allethrin or cyclethrin, but to show primarily the comparative effectiveness of cyclethrin and allethrin when each was combined with a synergist at a single ratio. The four groups of tests were not paired directly in the same test series. The greater degree to which cyclethrin is synergized by all four synergists for kill of house flies is obvious. Greater knockdown, also, is noted for cyclethrin with the four synergists.

A more detailed comparison was made with synergists sulfoxide and

piperonyl butoxide each with cyclothrin and pyrethrins. Four ratios of synergist to toxicant at three different dosage levels of cyclothrin or pyrethrins were tested. Results for sulfoxide and piperonyl butoxide are shown in Tables III and IV respectively. Data for 10-minute knockdown

TABLE IV

EFFECTIVENESS OF DIFFERENT RATIOS OF PIPERONYL BUTOXIDE WITH CYCLOTHRIN AND PYRETHRINS IN SPACE SPRAYS FOR KNOCKDOWN AND KILL OF HOUSE FLIES*

| Insecticide** | | Piperonyl butoxide** (mg.) | % Kill 24 hrs. | % Knockdown | | | OTI difference | |
|---------------|------------------------|----------------------------------|-------------------|-------------|--------|---------|-----------------|---------------------------|
| Name | Concentration (mg.) | | | 3 Min. | 5 Min. | 10 Min. | Kill 24 hrs. | Knock- down 10 min. |
| Cyclothrin | 25 | 100 | 40 | 57 | 69 | 81 | - 1 | -14 |
| | 25 | 200 | 53 | 64 | 75 | 87 | +12 | - 8 |
| | 25 | 300 | 52 | 66 | 76 | 87 | +11 | - 8 |
| | 25 | 400 | 57 | 64 | 76 | 86 | +16 | - 9 |
| | 50 | 100 | 55 | 70 | 83 | 92 | +14 | - 3 |
| | 50 | 200 | 65 | 74 | 84 | 92 | +24 | - 3 |
| | 50 | 300 | 76 | 78 | 89 | 95 | +35 | 0 |
| | 50 | 400 | 84 | 75 | 88 | 96 | +43 | + 1 |
| | 75 | 100 | 75 | 79 | 89 | 95 | +34 | 0 |
| | 75 | 200 | 80 | 82 | 90 | 95 | +39 | 0 |
| | 75 | 300 | 88 | 83 | 91 | 96 | +47 | + 1 |
| | 75 | 400 | 93 | 84 | 93 | 98 | +52 | + 3 |
| Pyrethrins | 25 | 100 | 46 | 73 | 82 | 89 | + 5 | - 6 |
| | 25 | 200 | 59 | 78 | 88 | 93 | +18 | - 2 |
| | 25 | 300 | 66 | 81 | 91 | 95 | +25 | 0 |
| | 25 | 400 | 70 | 83 | 91 | 94 | +29 | - 1 |
| | 50 | 100 | 66 | 86 | 92 | 96 | +25 | + 1 |
| | 50 | 200 | 80 | 88 | 94 | 97 | +39 | + 2 |
| | 50 | 300 | 82 | 90 | 95 | 98 | +41 | + 3 |
| | 50 | 400 | 90 | 89 | 95 | 97 | +49 | + 2 |
| | 75 | 100 | 81 | 90 | 94 | 97 | +40 | + 2 |
| | 75 | 200 | 91 | 91 | 95 | 98 | +50 | + 3 |
| | 75 | 300 | 94 | 93 | 96 | 98 | +53 | + 3 |
| | 75 | 400 | 97 | 94 | 97 | 98 | +56 | + 3 |
| OTI | | | 41 | 89 | 93 | 95 | | |

* Figures for synergist and toxicant are averages of 5 paired tests compared with 20 tests for the OTI.

** Mg. of chemical in 100 ml. of refined petroleum distillate.

and 24-hour mortality at the 200-mg. dosage of synergist were transposed to a logarithmic probability scale. Then the following amounts of pyrethrins or cyclothrin each with 200 mg. of synergist, to produce a spray which equaled the Official Test Insecticide for 10-minute knockdown and which exceeded it by 20 percentage points for 24-hour mortality, were determined:

| Toxicant | Synergist | |
|--|--------------------|-----------|
| | Piperonyl butoxide | Sulfoxide |
| Pyrethrins, mg./100 ml. petroleum distillate | 25 | 27 |
| Cyclethrin, mg./100 ml. petroleum distillate | 40 | 46 |

These figures show that 1.6 and 1.7 times as much cyclethrin as pyrethrins are needed for comparable performance. At lower concentrations of the synergists these amounts of cyclethrin are slightly less and at high concentrations they are somewhat higher. The relative efficiencies of sulfoxide and piperonyl butoxide with cyclethrin and pyrethrins will be discussed in a forthcoming publication.

The amounts of cyclethrin and pyrethrins needed to provide high kill and knockdown when combined with fixed amounts of piperonyl butoxide and sulfoxide are shown in Table V. It has been demonstrated over a period of several months in the Peet-Grady tests that only 1.6 to 1.7 times as much cyclethrin as pyrethrins was needed with synergists piperonyl butoxide or sulfoxide to provide excellent Grade AA sprays. On the other hand, two times as much allethrin and two times as much piperonyl butoxide are needed to equal the pyrethrins sprays (Table VI).

TABLE V

EFFECTIVENESS OF TWO SYNERGISTS WITH PYRETHRINS AND CYCLETHRIN IN SPACE SPRAYS FOR KNOCKDOWN AND KILL OF HOUSE FLIES

| Formulation | | No. of tests | % Kill 24 hrs. | % Knockdown | | | OTI difference | |
|-------------------|---------------------|--------------|----------------|-------------|--------|---------|----------------|--------------------|
| Insecticide (mg.) | Synergist (200 mg.) | | | 3 Min. | 5 Min. | 10 Min. | Kill 24 hrs. | Knock-down 10 min. |
| Pyrethrins 25 | Piperonyl butoxide | 7 | 58 | 74 | 86 | 94 | +19 | -1 |
| Cyclethrin 40 | Piperonyl butoxide | 7 | 59 | 64 | 79 | 92 | +20 | -3 |
| Cyclethrin 45 | Piperonyl butoxide | 7 | 66 | 66 | 83 | 94 | +27 | -1 |
| Pyrethrins 25 | Sulfoxide | 7 | 68 | 79 | 89 | 96 | +29 | +1 |
| Cyclethrin 40 | Sulfoxide | 7 | 67 | 69 | 87 | 95 | +28 | 0 |
| Cyclethrin 45 | Sulfoxide | 7 | 71 | 74 | 87 | 96 | +32 | +1 |
| OTI | | 17 | 39 | 87 | 92 | 95 | | |

Oil sprays on male German cockroaches. The effect of cyclethrin, pyrethrins, and allethrin, without synergists, in oil sprays on adult German cockroaches was determined. The LD₅₀ values in 48 hours for pyrethrins, allethrin and cyclethrin in petroleum distillate were respectively 90, 330, and 540 mg./100 ml. Thus, cyclethrin with no synergist is less effective than allethrin or pyrethrins on these insects.

On the other hand synergized cyclethrin was found to be superior to allethrin (Table VII). This greater degree of synergism is especially evi-

TABLE VI

EFFECTIVENESS OF SYNERGISTS WITH PYRETHRINS, CYCLETHRIN, AND ALLETHRIN
IN SPACE SPRAYS FOR KNOCKDOWN AND KILL OF HOUSE FLIES

| Formulation | | No. of tests | % Kill 24 hrs. | % Knockdown | | | OTI difference | |
|----------------------|------------------------|--------------------|-------------------------|-------------|-----------|------------|--------------------|---------------------------|
| Insecticide (mg.) | Synergist (mg.) | | | 3 Min. | 5 Min. | 10 Min. | Kill 24 hrs. | Knock- down 10 min. |
| Pyrethrins 25 | Sulfoxide 200 | 18 | 73 | 87 | 93 | 96 | +38 | +1 |
| OTI | | 25 | 35 | 90 | 93 | 95 | | |
| Cyclethrin 42 | Sulfoxide 200 | 94 | 67 | 79 | 87 | 94 | +29 | -1 |
| OTI | | 68 | 38 | 91 | 93 | 95 | | |
| Allethrin 42 | Sulfoxide 200 | 8 | 39 | 80 | 86 | 91 | +2 | -4 |
| OTI | | 13 | 37 | 90 | 93 | 95 | | |
| Pyrethrins 25 | Piperonyl butoxide 200 | 47 | 67 | 83 | 90 | 95 | +28 | 0 |
| OTI | | 61 | 39 | 91 | 93 | 95 | | |
| Cyclethrin 42 | Piperonyl butoxide 200 | 83 | 65 | 75 | 85 | 94 | +27 | -2 |
| OTI | | 59 | 38 | 92 | 94 | 96 | | |
| Allethrin 42 | Piperonyl butoxide 200 | 8 | 45 | 76 | 83 | 90 | +8 | -5 |
| OTI | | 13 | 37 | 90 | 93 | 95 | | |
| Allethrin 50 | Piperonyl butoxide 400 | 5 | 59 | 82 | 90 | 95 | +22 | 0 |
| OTI | | 5 | 37 | 90 | 93 | 95 | | |

dent with sulfoxide mixtures. It can be seen in Table VII that 150 mg. of cyclethrin with synergist provides significantly higher kill and knockdown than allethrin. That is, 73 per cent and 45 per cent kills, respectively, and 41 per cent and 18 per cent knockdown were obtained. Piperonyl butoxide also synergized cyclethrin better than allethrin for knockdown. Neither

TABLE VII

EFFECTIVENESS OF PYRETHRINS, CYCLETHRIN, AND ALLETHRIN WITH SYNERGISTS IN SPACE
SPRAYS FOR KNOCKDOWN AND KILL OF ADULT MALE GERMAN COCKROACHES

| Formulation | | No. of repl. of 20 roaches | % Kill 48 hrs. | % Knock- down | OTI difference | |
|----------------------|------------------------|-------------------------------------|-------------------|---------------------|-----------------|--------------------------|
| Insecticide (mg.) | Synergist (mg.) | | | 15 Min. | Kill 48 hrs. | Knock down 15 min. |
| | | | | | | |
| (Series A) | | | | | | |
| Pyrethrins 50 | Piperonyl butoxide 500 | 16 | 79 | 78 | -14 | -18 |
| Pyrethrins 50 | Sulfoxide 500 | 15 | 83 | 76 | -10 | -20 |
| Cyclethrin 150 | Sulfoxide 750 | 15 | 73 | 41 | -20 | -55 |
| Allethrin 150 | Sulfoxide 750 | 10 | 45 | 18 | -48 | -78 |
| (Series B) | | | | | | |
| Pyrethrins 50 | Piperonyl butoxide 500 | 19 | 78 | 77 | -13 | -20 |
| Pyrethrins 50 | Sulfoxide 500 | 35 | 88 | 81 | + 3 | -13 |
| Cyclethrin 100 | Piperonyl butoxide 500 | 14 | 36 | 68 | -40 | -20 |
| Allethrin 100 | Piperonyl butoxide 500 | 14 | 45 | 36 | -31 | -52 |

the cyclothrin nor allethrin mixtures are equivalent to the synergized pyrethrins with piperonyl butoxide or sulfoxide.

Aerosols on house flies. Cyclothrin has been compared with allethrin and pyrethrins in low-pressure aerosol formulations. In one series of tests, the three materials were each tested with synergists, piperonyl butoxide and sulfoxide, at three ratios (8 to 1, 4 to 1, and 2 to 1) of synergist to toxicant. The concentration of synergist was constant at 1.6 per cent and the toxicant was varied at 0.2, 0.4, and 0.8 per cent. Eight paired tests were conducted with each formulation. The data are shown in Table VIII.

TABLE VIII

EFFECTIVENESS OF 1.6 PER CENT PIPERONYL BUTOXIDE OR SULFOXIDE WITH DIFFERENT CONCENTRATIONS OF PYRETHRINS, ALLETHRIN, AND CYCLETHRIN IN LOW-PRESSURE AEROSOLS FOR KNOCKDOWN AND KILL OF HOUSE FLIES*

| Insecticide** | | % Kill 24 hrs. | % Knockdown | | | OTA difference | | | |
|--------------------|------------|-------------------|-------------|------------|------------|-------------------|-----------|------------|------------|
| Synergist | Name | % Concn. | 5 Min. | 10 Min. | 15 Min. | % Kill 24 hrs. | 5 Min. | 10 Min. | 15 Min. |
| Piperonyl butoxide | Pyrethrins | 0.1 | 59 | 55 | 72 | 82 | -29 | +3 | +14 |
| Piperonyl butoxide | Pyrethrins | 0.2 | 74 | 63 | 81 | 90 | -14 | +12 | +22 |
| Piperonyl butoxide | Pyrethrins | 0.4 | 88 | 63 | 84 | 91 | -1 | +11 | +23 |
| Piperonyl butoxide | Pyrethrins | 0.8 | 94 | 73 | 90 | 96 | +6 | +21 | +28 |
| Piperonyl butoxide | Allethrin | 0.2 | 51 | 46 | 62 | 71 | -37 | -5 | +3 |
| Piperonyl butoxide | Allethrin | 0.4 | 59 | 61 | 71 | 83 | -30 | +10 | +14 |
| Piperonyl butoxide | Allethrin | 0.8 | 70 | 65 | 83 | 91 | -18 | +14 | +23 |
| Piperonyl butoxide | Cyclothrin | 0.2 | 62 | 55 | 71 | 84 | -27 | +4 | +16 |
| Piperonyl butoxide | Cyclothrin | 0.4 | 84 | 57 | 79 | 90 | -5 | +6 | +22 |
| Piperonyl butoxide | Cyclothrin | 0.8 | 95 | 69 | 87 | 95 | +6 | +18 | +27 |
| Sulfoxide | Pyrethrins | 0.1 | 59 | 50 | 68 | 78 | -29 | -2 | +10 |
| Sulfoxide | Pyrethrins | 0.2 | 80 | 61 | 82 | 93 | +1 | +9 | +25 |
| Sulfoxide | Pyrethrins | 0.3 | 87 | 63 | 81 | 91 | -1 | +12 | +23 |
| Sulfoxide | Pyrethrins | 0.4 | 97 | 69 | 90 | 96 | +9 | +17 | +28 |
| Sulfoxide | Allethrin | 0.2 | 45 | 46 | 60 | 69 | -43 | -6 | +1 |
| Sulfoxide | Allethrin | 0.4 | 61 | 61 | 77 | 87 | -27 | +9 | +18 |
| Sulfoxide | Allethrin | 0.8 | 75 | 67 | 83 | 91 | -14 | +16 | +23 |
| Sulfoxide | Cyclothrin | 0.2 | 62 | 56 | 71 | 79 | -27 | +5 | +11 |
| Sulfoxide | Cyclothrin | 0.4 | 74 | 61 | 78 | 90 | -14 | +9 | +22 |
| Sulfoxide | Cyclothrin | 0.8 | 94 | 68 | 86 | 95 | +5 | +17 | +27 |
| OTA*** | | | 88 | 51 | 63 | 68 | | | |
| OTI | | | 44 | | 96 | | | | |

* Eight tests were made with the test formulations compared with 32 tests for the OTA and the OTI.

** Ingredients are per cent by weight.

*** Official Test Aerosol contains 0.4 per cent pyrethrins and 2.0 per cent DDT.

These data were plotted on a logarithmic probability scale and values were interpolated as shown in Table IX. The results with OTA were taken as a reference point and then the amounts of pyrethrins, cyclothrin, and allethrin to equal this were taken from the graph. Pyrethrins were then given a value of one and the relative amounts of cyclothrin or allethrin to equal pyrethrins were determined. An average of 1.8 times as much cyclothrin and 3.7 to 5.8 times the amount of allethrin, depending on the synergist, were required to equal pyrethrins.

TABLE IX

SUMMARY OF RESULTS SHOWING EFFECTIVENESS OF SYNERGIZED PYRETHRINS, CYCLETHRIN, AND ALLETHRIN IN LOW-PRESSURE AEROSOLS FOR KNOCKDOWN AND KILL OF HOUSE FLIES

| Test | Relative amounts for comparable performance with pyrethrins | | |
|------------------------------|---|------------|-----------|
| | OTA (%) | Cyclethrin | Allethrin |
| Synergist sulfoxide | | | |
| 5 Minute knockdown | 51 | 1.6 | 3.1 |
| 10 Minute knockdown | 63 | 1.1 | 2.6 |
| 15 Minute knockdown | 68 | 2.4 | 3.8 |
| 24 Hr. kill | 83 | 2.0 | 5.2 |
| Average | | 1.8 | 3.7 |
| Synergist piperonyl butoxide | | | |
| 5 Minute knockdown | 51 | 1.8 | 2.4 |
| 10 Minute knockdown | 63 | 2.5 | 4.6 |
| 15 Minute knockdown | 68 | 1.5 | 3.8 |
| 24 Hr. kill | 83 | 1.2 | 9.3 |
| Average | | 1.8 | 5.8 |

One aspect of considerable interest and practical importance is the degree to which DDT improves synergized allethrin formulations as compared with cyclethrin mixtures. It has been shown that cyclethrin is superior to allethrin with synergists piperonyl butoxide and sulfoxide. However, when DDT is added to such mixtures, the performance of this type of an allethrin formulation is improved so it equals that of a corresponding cyclethrin formulation (Table X). Kill and knockdown were equal for piperonyl butoxide plus DDT with allethrin or cyclethrin. Results for piperonyl butoxide with no DDT combined with allethrin or cyclethrin showed the superiority of the latter for knockdown and kill. Similar comparisons can also be made with sulfoxide mixtures. The data given in Table X are typical of several aerosol formulations which have been assayed to determine their effectiveness on house flies.

Aqueous sprays on agricultural insects. Cyclethrin has been tested in the laboratory against several insects attacking farm crops. In preliminary tests, it has been shown that allethrin and cyclethrin without synergist in aqueous sprays are equal for kill of spider mite nymphs and adults and Mexican bean beetle larvae. However, cyclethrin is significantly more effective than allethrin on the bean aphid (Table XI).

Mixtures containing certain synergists activate cyclethrin to a greater extent than allethrin on these crop pests. However, synergized cyclethrin is not so effective as synergized pyrethrins. Field work is needed to deter-

TABLE X

EFFECTIVENESS OF CYCLETHRIN AND ALLETHRIN WITH SYNERGISTS IN PRESENCE AND ABSENCE OF DDT IN LOW-PRESSURE AEROSOLS FOR KNOCKDOWN AND KILL OF HOUSE FLIES*

| DDT | Formulation** | | No. of tests | % Kill 24 hrs. | % Knock-down 15 min. | OTA difference | |
|------|-----------------|---------------|--------------|----------------|----------------------|----------------|--------------------|
| | Insecticide (%) | Synergist (%) | | | | Kill 24 hrs. | Knock-down 15 min. |
| 2% | Cyclethrin 0.34 | Pip. but. 1.6 | 19 | 88 | 81 | - 4 | +13 |
| | Allethrin 0.34 | Pip. but. 1.6 | 19 | 92 | 80 | 0 | +12 |
| None | Cyclethrin 0.34 | Pip. but. 1.6 | 11 | 80 | 89 | -12 | +13 |
| | Allethrin 0.34 | Pip. but. 1.6 | 11 | 61 | 81 | -31 | + 5 |
| 2% | Cyclethrin 0.34 | Sulfoxide 1.6 | 19 | 90 | 82 | - 2 | +14 |
| | Allethrin 0.34 | Sulfoxide 1.6 | 19 | 91 | 81 | - 1 | +13 |
| None | Cyclethrin 0.34 | Sulfoxide 1.6 | 11 | 81 | 90 | -11 | +14 |
| | Allethrin 0.34 | Sulfoxide 1.6 | 11 | 68 | 82 | -24 | + 6 |

* Figures represent averages of 24 tests for formulations containing 2 per cent DDT and 16 tests for formulations with no DDT.

** Ingredients are per cent by weight. Aerosols contained in addition to above 42.5 per cent each of "Freons" 11 and 12, 6.5 per cent methylated naphthalenes and refined petroleum distillate to 100 per cent.

mine whether synergized cyclethrin would be effective on insects attacking agricultural crops.

Wheat protectants. During the last few years, it has been demonstrated that synergized pyrethrins impregnated on an inert dust diluent can be used to prevent insect attack on stored grain. In laboratory tests, allethrin is also effective when formulated with piperonyl butoxide and sulfoxide. In general, 20 and 25 per cent more allethrin and synergist respectively are needed to equal synergized pyrethrins mixtures.

Experiments were conducted with wheat using the rice weevil as the test insect. Cyclethrin was found to be equal to allethrin when the materials were used alone or with synergists. In this application cyclethrin has not been synergized to a greater degree than allethrin (Table XII).

TABLE XI

EFFECTIVENESS OF UNSYNERGIZED PYRETHRINS, CYCLETHRIN, AND ALLETHRIN FOR KILL OF THREE AGRICULTURAL INSECT PESTS

| Insecticide | Concn. in mg. per 100 ml. of water for LD ₉₅ | | |
|-------------|---|-----------------------------|----------------------------|
| | Bean aphids | Spider mite nymphs & adults | Mexican bean beetle larvae |
| Pyrethrins | 3.5 | 92 | 32 |
| Cyclethrin | 25 | 290 | 66 |
| Allethrin | 52 | 270 | 52 |

Livestock sprays. Cyclethrin has shown considerable promise in livestock sprays for control of flies attacking dairy cows. It has been compared with pyrethrins in oil-space spray and repellent-type mixtures, in water-based sprays and in treadle-sprayer concentrates. In these field experiments, cyclethrin was tested at the same dosage, and at 1.7 and 2.0 times that of pyrethrins. Two synergists, piperonyl butoxide and sulfoxide, and CRAG Fly Repellent (butoxypolypropylene glycol) were evaluated with pyrethrins and cyclethrin. Oil and water-based sprays were tested for repellency against stable, horn and house flies. Concentrates in treadle sprayers were evaluated against *Tabanus* species and stable flies.

The scope of these experiments is broad and detailed data cannot be

TABLE XII

EFFECTIVENESS OF CYCLETHRIN, ALLETHRIN, AND PYRETHRINS WITH SYNERGISTS
PIPERONYL BUTOXIDE AND SULFOXIDE IN DUST MIXTURES FOR KILL OF RICE
WEEVIL ON WHEAT

| Formulation* | | Concentration in milligrams of formulated dust for kill following seven-day exposure | |
|-----------------|------------------------|--|------------------|
| Insecticide (%) | Synergist (%) | LD ₅₀ | LD ₉₅ |
| Cyclethrin 0.1 | Piperonyl butoxide 1.6 | 64 | 130 |
| Allethrin 0.1 | Piperonyl butoxide 1.6 | 64 | 140 |
| Pyrethrins 0.08 | Piperonyl butoxide 1.1 | 55 | 150 |
| Cyclethrin 0.1 | Sulfoxide 1.6 | 58 | 130 |
| Allethrin 0.1 | Sulfoxide 1.6 | 60 | 170 |

* Ingredients are per cent by weight.

reported at this time. However, the over-all results indicate that 1.6 to 1.7 times more cyclethrin than pyrethrins, each with synergists, was needed for control of these fly species. This conclusion is based on the results of two seasons' work with oil sprays in New York, New Jersey and South Carolina, and one season's work in Oklahoma. Water sprays have been tested for two seasons in South Carolina and New Jersey and one season in New York and Oklahoma. Treadle-sprayer concentrates have been tested for one season in Illinois, Oklahoma and New Jersey.

DISCUSSION AND CONCLUSIONS

Certain considerations should be taken into account when comparing a new chemical such as cyclethrin with pyrethrins or allethrin. Pyrethrum has been shown to be highly effective against a broad group of insect pests. Its relatively high cost and poor residual insecticidal properties have curtailed its wider use and applications.

Allethrin is now going into its fifth year and experience has indicated it has been accepted widely for use in aerosols. Assuming that cyclethrin

can compete economically with allethrin it offers definite advantages over allethrin. This is summarized in part by noting relative use levels of allethrin and cyclethrin compared with pyrethrins in Table XIII. It should be pointed out that these conclusions or comparisons are based on many replicated experiments, but only in one laboratory. It is recognized that an insecticide of this nature has to be widely tested in many laboratories

TABLE XIII

APPROXIMATE AMOUNTS OF CYCLETHRIN AND ALLETHRIN NEEDED TO EQUAL PYRETHRINS WITH SYNERGISTS PIPERONYL BUTOXIDE AND SULFOXIDE FOR CERTAIN INSECTICIDE APPLICATIONS

| Application | Test method | Mg. in 100 ml. of petroleum distillate | | | |
|---|---|--|-----------------|-----------------|----------------|
| | | Syner- gist | Insecticide | | |
| | | | Pyre- thrins | Cycle- thrin | Alle- thrin |
| Household oil-space sprays to provide knockdown and kill of houseflies | Large group Peet-Grady | 200 | 25 | 42-50 | 75-100 |
| Contact oil sprays for German roaches | Official cockroach spray method | 500 | 50 | 200-300 | 300 |
| Oil sprays for use in dairy barns and on dairy cows | Large group Peet-Grady and field repellency tests | 200 | 25 | 42-50 | 75-100 |
| Concentrates when diluted in water for application to livestock to prevent attack from biting flies | Large group Peet-Grady and field repellency tests | 1000 | 100 | 150-200 | At least 200 |
| | | Relative amounts of insecticide when amount of synergist is constant | | | |
| Low-pressure aerosols without DDT | CSMA aerosol test method | — | 1 | 2 | 4 |
| Low-pressure aerosols with DDT | CSMA aerosol test method | — | 1 | 2 | 2 |
| Wheat protectants | Laboratory tests on rice weevils | — | 1 | 2 | 2 |

and under many varying conditions before the performance can be thoroughly evaluated with insecticides such as pyrethrins and allethrin.

The numerical values in Table XIII indicate cyclethrin has advantages over allethrin in oil-space sprays for flies and roaches. It has definite advantages in livestock repellent-toxicant type sprays as shown in replicated field experiments. Cyclethrin is twice as effective as allethrin in aerosols without DDT. When DDT is added, experimental results indicate this advantage is somewhat modified. In the laboratory, using rice weevils as the test insect, allethrin and cyclethrin are equivalent in wheat protectant-type formulations. Field experimentation is needed before the performance of cyclethrin can be evaluated against agricultural insect pests.

Extensive tests on warm-blooded animals have shown that the toxicity of cyclethrin is of the same nature as and no greater than that of allethrin and pyrethrins. By certain routes, it is considerably less toxic. This low order of mammalian toxicity is significant since insecticides of the pyrethrins and cyclethrin type come in intimate contact with man and animals and their food products.

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A METHOD OF FUMIGATING PLANTS WITH HYDROFLUORIC OR HYDROFLUOSILICIC ACID USING ATOMIZATION AND THERMAL ACTION¹

R. MAVRODINEANU

SUMMARY

Equipment and procedures are described for producing nearly constant concentrations of hydrofluosilicic acid (H_2SiF_6) and hydrofluoric acid (HF) in fumigation cabinets ranging from less than one to several hundred parts per billion. The method makes use of diluted solutions (0.01 to 1 per cent) of commercial preparations of these acids which are atomized by an air pressure of 10 to 12 lb. per sq. in. through a modified No. 15 De Vilbiss atomizer and are then passed through a low-temperature, electrically-heated tube where they are changed to the gaseous state before delivery to the air stream entering the fumigation cabinets. All equipment is of simple construction, relatively inexpensive, and has no moving parts.

In order to study the action of fluorine in the form of HF and H_2SiF_6 on plants, it is necessary to have a convenient cage constantly supplied with an air stream of controlled fluoride content. The first method employed at Boyce Thompson Institute made use of prepared dilute mixtures of HF and nitrogen injected through a flow meter into the air stream delivered to the fumigation cabinet. A more recent method involves the use of two carboys connected by a motor-driven metering pump. One of the containers is filled with mineral oil and the other with a mixture of nitrogen and gaseous HF in known amounts. When the pump introduces the mineral oil into the latter container, the gaseous mixture is pushed into the air duct supplying the fumigation cages. The concentration of fluoride in these cages can be largely varied by adjusting the concentration of the gas mixture or the pumping speed for a given air exchange.

This method, however, has several limitations. The carboys containing the gas mixture of nitrogen and HF are made of Pyrex glass which reacts with HF to produce silicon tetrafluoride (SiF_4). In the presence of water SiF_4 forms H_2SiF_6 . As a result the plants are treated with this latter acid instead of HF. Attempts to replace the glass containers with monel or polyethylene ones were unsuccessful.

The mineral oil used to displace the gas mixture absorbs HF and gradually releases it afterwards so that the concentration of the gas, expressed as HF, does not drop under approximately 1 per cent in the carboys. Under working conditions this means a concentration of 3 p.p.b. [parts per billion (10^9)] or less of fluorine, expressed as HF, in the cages. Thus the treatment of plants with concentrations of 3 p.p.b. or less has certain limitations.

¹ This article was preprinted October 1, 1954.

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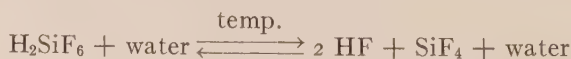
The Pyrex carboys used in the setup have a capacity of 45 liters which is sufficient to fumigate continuously for a period of nine days. When the apparatus is placed outdoors it is subjected to temperature and pressure variations which affect its functioning, depending upon the size of the carboys. As a result, plants are not treated with a constant concentration of fluoride but rather with a concentration which varies according to temperature and pressure changes.

Considering these facts it was desirable to find a method which permitted continuous treatment of plants with volatile fluorides in concentrations varying from less than one to several hundred parts per billion by volume, without being influenced appreciably by temperature and pressure changes. The first attempts were focused on generating HF gas by heating sodium bifluoride ($\text{NaF} \cdot \text{HF}$) pellets, a procedure which was suggested by Mr. E. J. Largent of Kettering Laboratories. A generator consisting of a copper cylinder of 300-ml. capacity provided with outlets at both ends was filled with $\text{NaF} \cdot \text{HF}$ pellets and heated by means of an electric heating jacket controlled by a powerstat-ammeter hookup. A continuous stream of dry nitrogen (of several liters per minute) controlled by a flow meter carried the gas from the generator to the air duct supplying the fumigation cage. The results obtained by means of this procedure were satisfactory for supplying a relatively low concentration (4 to 6 p.p.b. HF) at a temperature of 80°C . At higher temperatures the generation of HF was more difficult to control. Small temperature changes, between 100° and 150°C ., produced considerable variation in HF production.

A method was finally developed whereby the concentration of HF or H_2SiF_6 was more nearly controlled. The results obtained by this method justify a description of the equipment and the procedures followed.

PRINCIPLES, DESCRIPTION, AND OPERATION OF THE FLUORIDE GENERATOR

By this method the gas volume necessary for fumigation is obtained from a solution of H_2SiF_6 or HF diluted according to the desired fluoride content in the air. By means of a pneumatic atomizer the solution is transformed into fine droplets (mean diameter approximately 10μ) and sent into a low-temperature oven where the water is evaporated, and the acid passes from liquid to the gaseous state. From the oven the gas is introduced into the air stream supplying the fumigation cages. Where H_2SiF_6 is concerned, decomposition also takes place along with the evaporation according to the following reversible reaction:



which proceeds from left to right in the low-temperature oven and from right to left at the outlet from the oven. For the H_2SiF_6 treatment the

whole setup is made of Pyrex; for the HF it has to be made of a material resistant to corrosion as, for instance, polyethylene.

Figure 1 illustrates the equipment used to dispense the gases. The atomization container C is composed of a conical Pyrex flask of 2000-ml. capacity, provided with a superior lateral tube for droplet evacuation and an inferior lateral tube through which the liquid resulting from the condensation of big droplets on the walls of container C is recovered in container B. The No. 15 De Vilbiss atomizer was modified as shown at E and attached to the atomization container by means of a rubber stopper. An air pressure of 10 to 12 lb. per sq. in. proved satisfactory for this atomizer. Compressed air from the laboratory line was reduced by means of a pressure regulator connected with an air filter (not shown in Fig. 1). The

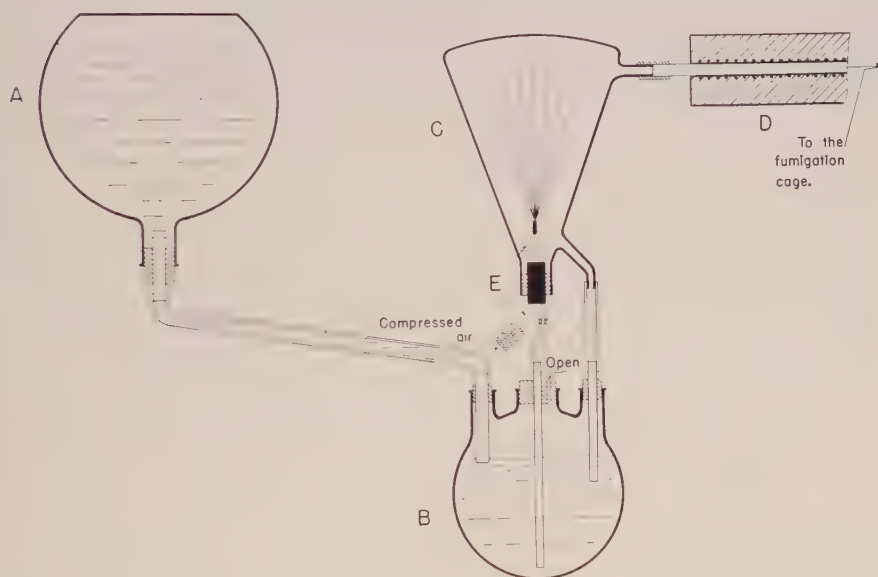


FIGURE 1. Constant fluoride concentration dispensing unit. (Explanation in text.)

atomizer is supplied with the solution through a central tube lowered to the bottom of the three-neck flask B which has a capacity of 3000 ml. When atomization is started by operating the air valve, the solution is sucked from flask B into the atomizer and atomized in container C. The mist of fine droplets passes into the low-temperature oven, D, while the larger droplets are condensed on the wall of the atomization container C and returned to B, the liquid being thus recirculated. In order to compensate automatically for the liquid used in the atomization process, a constant leveling device (flask A) is provided which consists of a round flask of 6000-ml. capacity connected to container B through a tygon tube. The same plastic material is used for the other connections. In a day 400 ml. of

solution are dispensed by the atomizer. As a result of the atomization, part of the solution is sent through the low-temperature oven while part of it loses water by evaporation. Thus the concentration of the solution increases in container B. To compensate for this, a lower concentration is used in A. In this case the concentration of the solution in A is 20 per cent of that in B. These values are determined empirically and depend on the working conditions, such as atomizer type, air volume, etc., and should be determined for each setup.

The low-temperature oven is made of a copper tube of 10-mm. diameter and 1.80-m. length. Electrical heating is accomplished by use of a nichrome jacket coiled around the entire length of the tube and insulated both thermically and electrically with asbestos. In this case the nichrome wire measures 6.20 meters with a resistance of 2.120 ohms per ft., which is sufficient to produce a temperature of 150° C. inside the tube with an intake of 250 w. (110 v. and 2.3 amp.).

RESULTS AND DISCUSSION

The results obtained in fumigation experiments involved a cage of 200-cu. ft. capacity with one air exchange per minute.

A 1 per cent solution of H_2SiF_6 in flask B (prepared from a commercial 30 per cent reagent) gave in the cage a concentration of 70 to 100 p.p.b. HF by volume, 0.1 per cent H_2SiF_6 gave 8 to 11 p.p.b., and 0.01 per cent H_2SiF_6 gave 0.6 to 0.8 p.p.b. Various desired concentrations can be obtained by starting from HF or H_2SiF_6 solutions of corresponding concentrations.

The described setup operated for 15 days without interruption with a loading of 6000 ml. in container A and 3000 ml. in container B. The variation of concentration measured in the cage during this time was 0.6 to 0.8 p.p.b. expressed as HF by volume for a variation of the surrounding temperature from 19° to 38° C. It seems that temperature has a less important influence here than in the case of the glass carboy setup. However, one could expect such an influence if the volume of air enclosed in container A would warm up. In this case the solution from A would be pushed in B, thus diluting the one contained here. These eventual changes, as well as the control of the initial concentration of the solution, can be checked easily by running a simple acid-base titration in the presence of phenolphthalein as indicator, using sodium hydroxide and a known volume of HF solution at room temperature and at the boiling point for the H_2SiF_6 solution. Important temperature changes could also affect the atomization rate (superficial tension). So far no serious interference of this nature has been observed.

It is believed that this apparatus may be used for dispensing other volatile substances, such as hydrochloric and hydrobromic acids, etc.

EFFECT OF SUBFREEZING TEMPERATURES ON VIABILITY OF CONIFER SEEDS IN STORAGE

LELA V. BARTON

SUMMARY

Seeds of Ponderosa pine (*Pinus ponderosa* Dougl.), Douglas fir (*Pseudotsuga taxifolia* Britt.), Sitka spruce (*Picea sitchensis* Carr.), Western Red cedar (*Thuja plicata* Donn.), and Western hemlock (*Tsuga heterophylla* Sarg.) were stored in canvas bags at subfreezing temperatures of approximately -4° , -11° , and -18° C. for three years. It was found that -18° C. was best for maintaining viability and that deterioration was most rapid at -4° C.

INTRODUCTION

It has been known for some time that low temperatures are effective in prolonging the life span of conifer seeds. However, the superiority of subfreezing temperatures has been demonstrated more recently. Some of these effects and the literature on the subject have been described in a recent paper (1).

The present tests in which three different subfreezing temperatures were used, grew out of a need for the best possible storage condition for valuable, short-lived conifer seeds. Data from this laboratory had already furnished comparisons of -4° and 5° C., and Isaac (3) had stored Noble fir seed for five years at 15° F. In personal correspondence, Isaac also reported highly successful results of limited tests in which conifer seeds were held at temperatures down to 0° F., but no controlled experiments had been done to determine whether the degree of cold in the subfreezing range would affect viability.

MATERIALS AND METHODS

The seeds used for these investigations were those of Ponderosa pine, Douglas fir, Sitka spruce, Western Red cedar, and Western hemlock. They were all secured from the Pacific Northwest Forest Experiment Station, Portland, Oregon, through the courtesy of Mr. Leo A. Isaac, Forester. They were held at temperatures of freezing or below from the time of collection until they were shipped to Boyce Thompson Institute, where they were received on February 21, 1951. They were kept further at approximately -4° C. until April 16, 1951 when the present tests were begun.

Storage. The available seeds of each species were divided into three lots which were stored in canvas bags at temperatures of approximately -4° , -11° , and -18° C. For the first two temperatures, cold rooms with average temperatures as indicated were used. However, the temperatures of these rooms fluctuated $\pm 5^{\circ}$ C. A food freezer furnished the more constant temperature of -18° C.

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Germination. Viability tests were made at the beginning of the experiment and after 1, 2, and 3 years of storage. For germination the seeds, in duplicate lots of 100 each, were mixed with moist granulated peat moss and placed at 5° C. for one month after which they were transferred to 20° C. where most of the seedlings appeared within a period of one week. However, additional germinations were recorded for three additional weeks.

RESULTS AND DISCUSSION

The effect of the three low temperatures on the keeping quality of the seeds is shown in Table I. There was a great variation in the viability of the

TABLE I
GERMINATION AND MOISTURE CONTENT OF CONIFER SEEDS AFTER
STORAGE AT SUBFREEZING TEMPERATURES

| Species and per cent germination at time of storage | Approximate storage temp. (° C.) | Per cent germination after storage for | | | Per cent moisture after 3 yrs. storage |
|---|--|---|--------|--------|---|
| | | 1 yr. | 2 yrs. | 3 yrs. | |
| Ponderosa pine 95 | - 4 | 87 | 91 | 92 | 15 |
| | - 11 | 85 | 79 | 70 | 17 |
| | - 18 | 81 | 75 | 72 | 10 |
| Douglas fir 88 | - 4 | 69 | 44 | 7 | 15 |
| | - 11 | 85 | 75 | 60 | 16 |
| | - 18 | 88 | 80 | 78 | 10 |
| Sitka spruce 56 | - 4 | 49 | 11 | 1 | 13 |
| | - 11 | 62 | 38 | 25 | 12 |
| | - 18 | 60 | 55 | 47 | 9 |
| Western Red cedar 60 | - 4 | 66 | 27 | 9 | 17 |
| | - 11 | 65 | 31 | 24 | 17 |
| | - 18 | 74 | 74 | 76 | 12 |
| Western hemlock 12 | - 4 | 5 | 0 | 0 | 8 |
| | - 11 | 7 | 0 | 0 | 12 |
| | - 18 | 19 | 13 | 13 | 8 |

different species at the beginning of the experiment. Ponderosa pine and Douglas fir seeds were of excellent quality, giving 95 and 88 per cent germination, respectively, at the time the storage tests were begun. Sitka spruce and Western Red cedar were less vigorous with initial germination percentages of 56 and 60, while only 12 per cent of the Western hemlock seeds germinated.

All of the seeds, except those of Ponderosa pine, kept better at - 18° C. than at - 11° C. and deterioration was most rapid at - 4° C. (Table I). It was anticipated that some such effect might be obtained, but the rapidity of the response was striking. Definite differences in deterioration rates were evident after only two years of storage. In experiments with other

seeds (1) at least five years of storage were required to show differences in response to 5° and -4° C. The germination capacity of Douglas fir and Sitka spruce seeds after storage at the three temperatures is shown graphically in Figure 1.

Since none of these seeds were stored in sealed containers, samples were taken for moisture determination at the end of three years of storage. The seeds were introduced into the weighing bottles at the low temperatures so there was no chance of water condensation on the cold seeds. Drying was

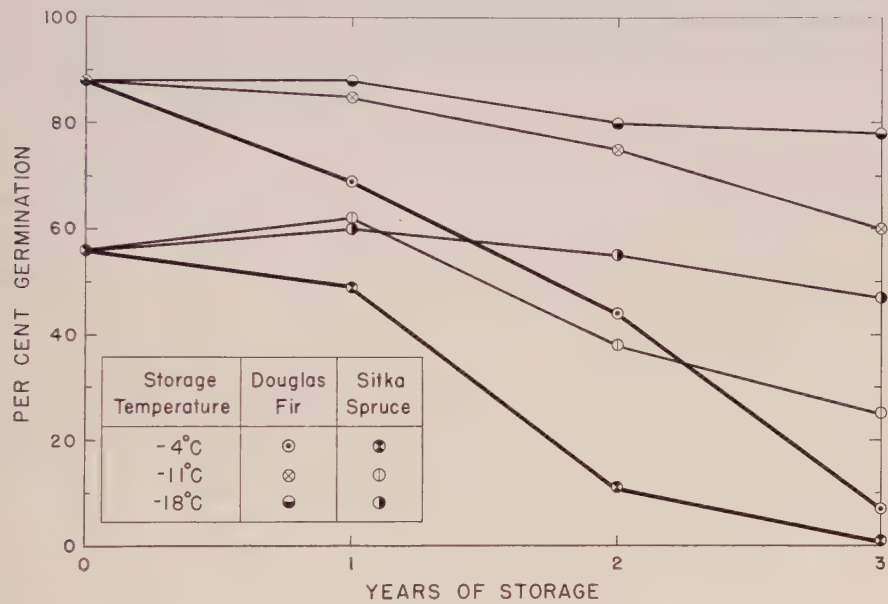


FIGURE 1. Germination of Douglas fir and Sitka spruce after storage at subfreezing temperatures.

in a vacuum oven at 80° C. for 48 hours. Moisture percentages were calculated on the basis of dry weight of seeds. In all cases, less moisture was present in the seeds at the lowest temperature (Table I). However, in view of the similarity of moisture contents in the seeds stored at -4° and -11° C., and the difference in keeping quality at these temperatures, it is believed that the more important effect under the conditions of this experiment was that of temperature.

It should be kept in mind that seeds were placed in open storage only. Other tests (2) have shown that reduction in moisture content extends the life of Douglas fir and Western hemlock seeds even at the very favorable temperature of -18° C.

It would seem, then, that the lower the subfreezing temperature (to

-18° C.), the better the survival of these conifers in storage. The behavior of seeds packeted after storage at -18° C. is described in another report (2). The present tests are being continued.

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STORAGE AND PACKETING OF SEEDS OF DOUGLAS FIR AND WESTERN HEMLOCK

LELA V. BARTON¹

SUMMARY

Data are given on the effect of different moisture contents of the seeds of Douglas fir (*Pseudotsuga taxifolia* Britt.) and Western hemlock (*Tsuga heterophylla* Sarg.) on their viability when stored at 5° C. and -18° C., and on the keeping quality of such stored seeds when packeted and kept for a further storage period at 5° C. or 30° C.

At moisture contents of 5.8 or 13.6 per cent in sealed storage at -18° C., and at a moisture content of 5.8 per cent at 5° C., full viability of Douglas fir seeds was retained for a period of three years. Similar effects were found for Western hemlock seeds with 7.7 and 11.0 per cent moisture. Douglas fir seeds in open storage at 5° and -18° C., and in sealed storage with 13.6 per cent moisture at 5° C. showed deterioration after 12 months, with the last named showing more marked deterioration than the other two after 24 months of storage. Open storage at 5° C. was more deleterious for Western hemlock seeds than for those of Douglas fir.

Sealed tin cans were best for packeting when the moisture content of the seeds was low, but not as good as Manila envelopes for high-moisture seeds. Storage for as long as 24 months at -18° C. had no harmful effects on seeds when they were packeted and held subsequently for six months. Deterioration in packets was more rapid at 30° than at 5° C., and for seeds with high-moisture than for those with low-moisture contents. Vinyl-laminated aluminum foil was effective for packeting seeds with low-moisture content to be stored at either 5° or 30° C., but caused rapid deterioration of seeds with high-moisture content when the storage was at 30° C. Seeds of both Douglas fir and Western hemlock stored for an extended time at -18° C. were more resistant to further unfavorable storage than those stored at 5° C. because of the higher germination capacity of the former.

INTRODUCTION

Seeds of Douglas fir and Western hemlock present a problem to the seedsman and forester on at least three counts. First of all, frequent seed crop failures make it difficult to maintain a constant supply of seeds for planting. Douglas fir trees, for example, produce a good crop every 3 to 7 years, with light crops in between. The set of good seeds in cones of Western hemlock is also likely to be sporadic. Another factor in the availability of seeds of these two species is their relatively short life span under ordinary conditions of storage (6, 7). Still another problem, related to the second one above, is that of retention of viability under shipping conditions. All of these difficulties are magnified by the tremendous importance

¹ The author wishes to express her appreciation to Manning Seed Company, Seattle, Washington for supplying seeds and financial support, in part, for this investigation.

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of these trees, especially Douglas fir, for timber, and the consequent large demand for seeds.

It has been demonstrated by Barton (4) and others that subfreezing storage is better than temperatures above freezing for maintaining the viability of many different kinds of seeds, including conifers. Furthermore, a more recent work (5) has shown that -18° C. is better than -11° or -4° C. for maintaining the viability of certain conifer seeds, including the two species considered here.

Some seed companies now use subfreezing storage rooms, but the question of the behavior of the seeds upon removal from the cold storage and packaging for shipment, and their ability to survive the shipment itself, have been matters of concern. Also, some customers have been reluctant to purchase so-called "frozen" seeds.

It has been shown for onion seeds (3) that the best condition for maintaining viability, as measured by testing immediately upon removal from storage, is the condition which permits the survival of the greatest number of seeds during periods of subsequent storage. However, detailed results of such controlled tests on the two seeds in question have not been available up to this time.

MATERIALS AND METHODS

Seeds of Douglas fir and Western hemlock of the 1950 crop were received in March 1951. They had been kept at 0° F. from the previous December and were shipped to this Institute by air express.

STORAGE

Two temperatures, 5° C. and -18° C., were used. Storage at 5° C. was in a large room with fairly dry atmosphere for the first two years but much more humid after that time. Subfreezing, -18° C., was secured in a food freezer. Both temperatures were constant, varying only $\pm 1^{\circ}$ C. Seeds with two different moisture contents were used.

When received, Douglas fir seeds contained 5.8 per cent moisture, calculated on the basis of the dry weight of the seeds. Some samples were stored without any moisture adjustment. In other samples, the moisture content was increased to 13.6 per cent by spreading the seeds in a very humid room at 5° C., where they absorbed the desired amount of water in three days. They were then placed in a sealed container at -4° C. for one week in order to ensure uniform distribution of the moisture increase throughout all the seeds of the lot. Samples of the seeds with 5.8 per cent moisture were placed in canvas bags for storage at 5° C. and -18° C. Other samples of the same lot, as well as of the lot with moisture content increased to 13.6 per cent, were stored at the same temperatures after having been placed in tin cans with tight-fitting lids, further sealed with seal-

ing wax. Each sample consisted of approximately 90 grams (about 8,400 seeds), one sample to be used for packeting tests after each original storage interval.

Moisture determinations on seeds of Western hemlock upon receipt indicated that they contained 11 per cent. One lot was stored without any adjustment and another was dried to a moisture content of 7.7 per cent by spreading the seeds in the laboratory for four hours. Original storage in canvas bags and sealed containers at 5° C. and -18° C. was as described above for Douglas fir.

All moisture determinations were made by drying the seeds in a vacuum oven at 75° C. for 48 hours. Under sealed storage, the initial moisture contents were maintained for the entire test period. The seeds stored in canvas bags were subjected to moisture fluctuations corresponding to the humidity of the surrounding atmosphere. At the end of two years of storage, all lots in canvas bags at both -18° C. and 5° C. contained approximately 13 per cent moisture. Subsequently, the atmosphere in the 5° C. room became much more humid, resulting in a moisture content of 22 to 26 per cent by the end of the third year of storage. At -18° C., the seeds still had about 13 per cent moisture after three years.

PACKETING

Samples of both species were removed from the original storage conditions after 6, 12, 18, and 24 months and packeted in Manila envelopes, foil envelopes, and tin cans for further storage at 5° and 30° C. Foil envelopes were made of vinyl-coated aluminum foil sealed by heat. Tin cans were sealed by tight-fitting lids and sealing wax.

Storage of the 5° C. packets was in a large room, the atmosphere of which was dry at the beginning of the test. When the atmosphere of this room became humid at the end of two years (see "Storage" above) all 5° C. packets were moved to a small, dry chamber maintained at this temperature. Storage of packets at 30° C. was in a dry room with good air circulation, resulting in a low moisture content (about 4.5 per cent) of the seeds in Manila envelopes.

Tests of the germination capacity of the seeds were made at the time of removal from the original storage conditions for packeting, and after 0.5, 1, 2, 3, 6, 12, and, in some cases, 18 and 24 months in the packets.

GERMINATION

Some of the factors affecting the germination of Douglas fir and hemlock seeds have been reported by Allen (1) and Baldwin (2).

In the present experiment it was necessary to determine a test method which would permit reliable comparisons after various treatments and years of storage. Seeds were pretreated in moist granulated peat moss at

1°, 5°, and 10° C. for periods of two weeks, one month, and six weeks. Some of the cultures were then transferred to 20° C. for germination. Others were planted in soil and placed in the greenhouse for seedling production. It soon became apparent that the greenhouse germination was unreliable due to variation in the temperature during the spring and summer months. As a result, later tests were made at 20° C. only. After extended pretreatment tests, including presoaking, it was found that viability could best be determined by mixing the seeds with granulated peat moss and leaving them at 5° C. for two weeks; then transferring them to 20° C. where the peak of germination was reached in 11 days, and practically complete germination took place in 18 days. As a standard procedure, all cultures were kept for three weeks after their transfer from 5° to 20° C. The germination data from duplicate lots of 100 seeds each, reported in this paper were secured by this method, which proved adequate for seeds of both Douglas fir and Western hemlock.

Cutting tests of 8 lots of 50 seeds each of Douglas fir and Western hemlock showed that 98 and 87 per cent were filled. However, the germination capacities of the seeds when received in this laboratory, as measured by the standard procedure just described, were only 85 and 49 per cent for Douglas fir and Western hemlock respectively.

RESULTS

RETENTION OF VIABILITY UNDER ORIGINAL STORAGE CONDITIONS

Douglas fir. The retention of viability under open and sealed conditions of storage at -18° and 5° C. for periods up to 36 months is shown in Figure 1 and in Table I (column 5). It will be recalled that 85 per cent of these seeds germinated at the time of storage. Germination tests were made after 6, 12, 18, and 24 months at the time samples were removed from the original storage conditions for packeting. Tests were also made after 36 months in original storage, though no seeds were packeted at that time.

Deterioration of seeds in open storage at either -18° or 5° C. became evident after one year, but after 24 months loss of viability was much more rapid at 5° C. than at -18° C. Seeds with 13.6 per cent moisture in sealed containers were also adversely affected at 5° C. Reduction of the moisture content to 5.8 per cent, followed by sealed storage, permitted the maintenance of full viability for 36 months at both -18° and 5° C. Also, -18° C. proved satisfactory for the storage of seeds with the higher moisture content of 13.6 per cent for the same length of time. It should be kept in mind that these are the results of testing the germination capacity of the seeds immediately upon removal from the original storage conditions, and do not necessarily reflect their behavior upon subsequent storage in packets.

Western hemlock. The effect of the original storage conditions upon the

TABLE I
EFFECT OF AGE AND STORAGE CONDITIONS ON THE SURVIVAL OF DOUGLAS FIR SEEDS IN PACKETS AT 30° C.

| Original Storage | | | Per cent germination after further storage in packets at 30° C., months | | | | | | | | | | | | | | | | | | | | | |
|------------------|---------------|----------------------|---|----------|-----------------|----------|----------|----------|----------|----------|----------|----------|----------|---------|----------|----------|---------------|----------|----|----------|----|----|--|--|
| Mos. | Temp., °C. | Open or sealed | % Moisture | o | Manila envelope | | | | | | Tin can | | | | | | Foil envelope | | | | | | | |
| | | | | | 0.5 | 1 | 2 | 3 | 6 | 12 | 0.5 | 1 | 2 | 3 | 6 | 12 | 0.5 | 1 | 2 | 3 | 6 | 12 | | |
| 6 | -18 | O | — | 81 | 82 | 79 | 83 | 84 | 62 | 10 | 81 | 77 | 74 | 54 | 5 | 13 | 83 | 59 | 20 | 0 | 0 | | | |
| | | S | 5.8 13.6 | 83 82 | 79 87 | 86 77 | 68 64 | 21 17 | 87 83 | 80 78 | 87 55 | 79 32 | 77 6 | 22 6 | 80 77 | 85 63 | 89 10 | 0 | 0 | 69 18 | | | | |
| | | S | — | 82 | 87 | 83 | 79 | 65 | 17 | 17 | 83 | 78 | 55 | 32 | 6 | 0 | 77 | 63 | 10 | 0 | 0 | | | |
| 12 | 5 | O | — | 73 | 70 | 80 | 81 | 67 | 50 | 10 | 78 | 76 | 60 | 36 | 5 | 1 | 69 | 53 | 55 | 69 | 0 | | | |
| | | S | 5.8 13.6 | 88 80 | 79 84 | 77 62 | 65 9 | 13 9 | 79 76 | 76 75 | 88 53 | 79 14 | 63 2 | 18 2 | 80 74 | 82 55 | 84 5 | 79 | 66 | 0 | 11 | | | |
| | | S | — | 77 | 69 | 70 | 76 | 75 | 45 | 19 | 71 | 69 | 73 | 64 | 27 | 3 | 60 | 46 | 3 | 0 | — | | | |
| 18 | 5 | O | — | 66 | 57 | 61 | 63 | 40 | 27 | — | 50 | 51 | 50 | 24 | 6 | 0 | 50 | 30 | 4 | 0 | — | | | |
| | | S | 5.8 13.6 | 75 62 | 73 55 | 76 68 | 56 52 | 53 34 | — | 72 58 | 72 51 | 76 60 | 63 25 | 69 | 9 | 75 43 | 67 30 | 69 | 51 | 59 | 3 | | | |
| | | S | — | 62 | 57 | 55 | 68 | 52 | 34 | 3 | 58 | 51 | 60 | 25 | 12 | 0 | 43 | 30 | 0 | 0 | — | | | |
| 18 | -18 | O | — | 49 | — | 57 | — | 49 | 49 | 19 | — | 20 | — | 13 | 30 | 0 | — | 4 | — | — | — | | | |
| | | S | 5.8 13.6 | 76 75 | — | 84 | — | 78 | 26 | 19 | — | 78 | — | 80 | 76 | 38 | — | 80 | — | 79 | 26 | | | |
| | | S | — | 75 | — | 81 | — | 77 | 69 | 19 | — | 69 | — | 64 | 65 | 8 | — | 53 | — | 0 | 0 | | | |
| 24 | 5 | O | — | 60 | — | 41 | — | 28 | 6 | 1 | — | 9 | — | 7 | 0 | — | — | 0 | — | — | — | | | |
| | | S | 5.8 13.6 | 79 59 | — | 75 | — | 82 | 71 | 19 | — | 72 | — | 72 | 66 | 14 | — | 72 | — | 70 | 24 | | | |
| | | S | — | 59 | — | 44 | — | 49 | 15 | 0 | — | 21 | — | 0 | — | — | — | 10 | — | 0 | — | | | |
| 24 | -18 | O | — | 50 | 53 | 38 | 55 | 53 | 37 | 21 | 48 | 26 | 31 | 25 | 15 | 7 | 35 | 45 | 1 | 0 | — | | | |
| | | S | 5.8 13.6 | 79 74 | 84 77 | 72 61 | 76 70 | 73 66 | 46 39 | 83 67 | 74 48 | 79 74 | 72 53 | 71 | — | 78 64 | 72 27 | 87 21 | 75 | 71 | — | | | |
| | | S | — | 74 | 77 | 61 | 70 | 66 | 69 | 36 | 67 | 48 | 74 | 53 | 4 | — | 64 | 27 | 21 | 4 | — | | | |
| 24 | 5 | O | — | 17 | 16 | 13 | 6 | 7 | 3 | 1 | 5 | 1 | 2 | 1 | — | 2 | 6 | 0 | 0 | — | — | | | |
| | | S | 5.8 13.6 | 73 42 | 69 33 | 64 37 | 78 40 | 71 28 | 45 12 | 36 | 67 26 | 54 13 | 61 10 | 59 | 60 | 19 | 67 26 | 53 | 69 | 62 | 16 | | | |
| | | S | — | 42 | 33 | 37 | 40 | 28 | 12 | 2 | 26 | 13 | 10 | 2 | 0 | 0 | 26 | 3 | 39 | 0 | — | | | |

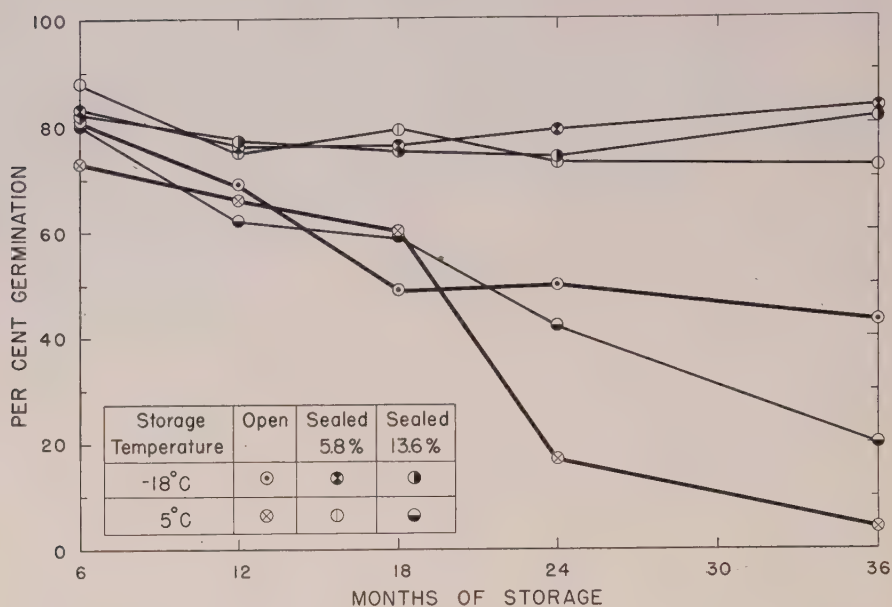


FIGURE 1. Germination capacity (per cent) of Douglas fir seeds stored at -18° or 5° C. in open or sealed containers. The moisture content of seeds in sealed containers was 5.8 or 13.6 per cent.

germination capacity of these seeds is shown in Table II (column 5). Although they were not so vigorous at the time the experiment was started (49 per cent germination), they did not deteriorate appreciably when held under the conditions favorable for Douglas fir. Deterioration of Western hemlock seeds in a canvas bag at 5° C., however, proceeded rapidly so that after 6, 12, and 18 months only 20, 11, and 2 per cent respectively germinated, whereas full viability of Douglas fir was maintained under these conditions for these shorter storage periods. Thus, for the first 18 months of open storage, a subfreezing temperature is more important for Western hemlock seeds than for those of Douglas fir. This was not apparent when the former were in sealed storage, especially with 7.7 per cent moisture, where they kept well at both 5° and -18° C.

RETENTION OF VIABILITY IN PACKETS

At 30° C. Because deterioration in packets was more rapid at 30° C. than at 5° C., a better measure of the length of the original storage period necessary to differentiate between -18° and 5° C. for keeping the seeds to prevent subsequent deterioration was secured for seeds in packets stored at 30° C. The effect of such packeting on Douglas fir seeds is shown in Table I and Figures 2 and 3. Considering first the seeds packeted in Manila

envelopes after original storage periods of 6, 12, or 24 months in a canvas bag at either $-18^{\circ}\text{C}.$ or $5^{\circ}\text{C}.$, it will be seen (Table I and Fig. 2) that the germination capacity of the seeds at the time of removal from the original storage condition for packeting was maintained at a fairly constant level for three months in the Manila envelopes. After 6 months in the packets there was a decline in viability which became serious after 12 months. In Figure 2, the importance of the subfreezing temperature for maintenance of high viability in original storage before packeting is apparent. Some difference was demonstrated in the 6- and 12-month periods of original storage, but this became much more marked after 24 months.

To see the effect of original storage on subsequent deterioration in cases where the initial moisture content of the seeds was maintained from the beginning, the germination behavior of seeds sealed with 5.8 and 13.6 per cent moisture packeted in tin cans was studied. The results are shown in Table I and some of them are pictured graphically in Figure 3 A. Similar retention of viability is to be noted for the seeds with 5.8 per cent moisture. However, there was much more rapid deterioration of seeds with 13.6 per cent moisture packeted in tin cans than in Manila envelopes. This was to be expected for the tin cans held the high moisture in the seeds while the dry conditions of the $30^{\circ}\text{C}.$ room permitted moist seeds packeted in Manila envelopes to benefit from drying to about 4.5 per cent. The effect of the moisture content on seeds packeted in tin cans at $30^{\circ}\text{C}.$ after 12 months

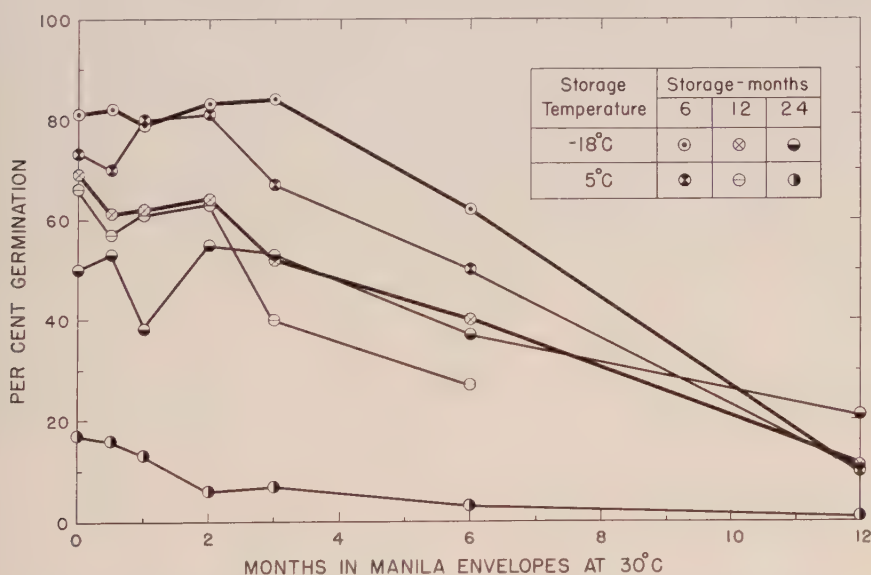


FIGURE 2. The effect of open storage at -18° and $5^{\circ}\text{C}.$ on the viability of Douglas fir seeds in Manila envelopes at $30^{\circ}\text{C}.$

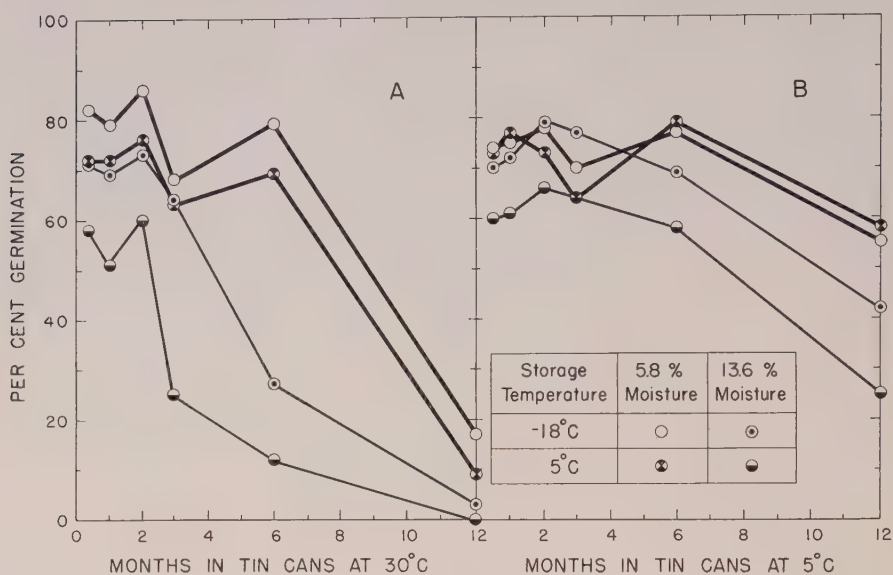


FIGURE 3. The effect of sealed storage of Douglas fir seeds at -18° and 5° C. for 12 months on their viability in tin cans held at (A) 30° C. and (B) 5° C.

original storage is pictured in Figure 3 A. Again, the superiority of -18° over 5° C., and of a moisture content of 5.8 over 13.6 per cent for original storage of Douglas fir seeds, is demonstrated. The advantage of the lower moisture content of 5.8 per cent, over 13.6 per cent, or that in canvas bags is seen in the resistance of the former to subsequent deterioration in packets at 30° C.

Tests under the dry conditions of the 30° C. room indicate that a Manila envelope is as good as a tin can for maintaining viability during further storage of seeds. The efficacy of the Manila envelope for packeting is no doubt due to the relatively dry conditions of the 30° C. room. Under high humidity conditions, the advantage of sealing seeds with low moisture content becomes apparent.

Except for the differences in initial viability and deterioration under original conditions of storage as noted above, the responses of Western hemlock seeds to packeting at 30° C. (Table II) were essentially the same as those of the Douglas fir.

At 5° C. The germination capacity of Douglas fir seeds upon further storage in three different kinds of packets at 5° C. is shown in Table III. The viability of all seeds stored at -18° C. was the same after six months of storage, whether in canvas bags, or in sealed containers with 5.8 or 13.6 per cent moisture. This initial high viability was maintained in all packets, Manila envelope, foil envelope, or tin can, for a period of 12 months.

TABLE II
EFFECT OF AGE AND STORAGE CONDITIONS ON THE SURVIVAL OF WESTERN HEMLOCK SEEDS IN PACKETS AT 30° C.

| Original storage | | | Per cent germination after further storage in packets at 30° C., months | | | | | | | | | | | | | | | | | | | | |
|------------------|---------------|----------------------|---|----------------|-----------------|----|----|----|----|----|---------|----|----|----|----|----|---------------|----|----|----|----|----|---|
| Mos. | Temp., °C. | Open or sealed | % Moisture | 0 | Manila envelope | | | | | | Tin can | | | | | | Foil envelope | | | | | | |
| | | | | | 0.5 | 1 | 2 | 3 | 6 | 12 | 0.5 | 1 | 2 | 3 | 6 | 12 | 0.5 | 1 | 2 | 3 | 6 | 12 | |
| 6 | -18 | O S S | — 7.7 11.0 | 46 44 40 | 37 | 44 | 40 | 46 | 33 | 9 | 30 | 41 | 33 | 50 | 33 | 12 | 33 | 35 | 31 | 29 | 32 | 11 | |
| | | | | | 49 | 42 | 38 | 41 | 41 | 9 | 42 | 44 | 45 | 48 | 38 | 13 | 42 | 49 | 46 | 45 | 42 | 4 | 1 |
| | | | | | 41 | 43 | 35 | 42 | 36 | 12 | 45 | 35 | 32 | 33 | 38 | 10 | 41 | 33 | 37 | 11 | 0 | | |
| | 5 | O S S | — 7.7 11.0 | 20 51 42 | 13 | 17 | 18 | 15 | 8 | 1 | 14 | 10 | 11 | 8 | 10 | 0 | 13 | 4 | 1 | 0 | 1 | 0 | |
| | | | | | 45 | 50 | 49 | 49 | 38 | 11 | 39 | 49 | 48 | 43 | 45 | 16 | 41 | 41 | 43 | 51 | 41 | 14 | 0 |
| | | | | | 35 | 44 | 39 | 44 | 37 | 16 | 40 | 36 | 49 | 44 | 31 | 13 | 35 | 30 | 34 | 32 | 12 | | |
| 12 | -18 | O S S | — 7.7 11.0 | 40 51 44 | 30 | 31 | 30 | 36 | 26 | 15 | 36 | 39 | 28 | 26 | 22 | 16 | 33 | 14 | 15 | 1 | 0 | — | |
| | | | | | 51 | 40 | 37 | 41 | 30 | 23 | 50 | 47 | 37 | 35 | 21 | 39 | 43 | 39 | 39 | 18 | 9 | — | |
| | | | | | 44 | 46 | 33 | 30 | 30 | 22 | 46 | 37 | 33 | 33 | 29 | 24 | 37 | 36 | 28 | 10 | 0 | | |
| 18 | 5 | O S S | — 7.7 11.0 | 11 44 42 | 8 | 7 | 8 | 2 | 1 | 0 | 7 | 4 | 6 | 3 | 2 | 0 | 4 | 2 | 0 | 1 | 0 | — | |
| | | | | | 44 | 38 | 38 | 34 | 37 | 16 | 45 | 51 | 43 | 38 | 28 | 15 | 45 | 41 | 42 | 33 | 6 | 0 | |
| | | | | | 39 | 34 | 34 | 34 | 27 | 14 | 39 | 31 | 41 | 28 | 28 | 24 | 35 | 26 | 19 | 21 | 19 | | |
| | -18 | O S S | — 7.7 11.0 | 54 46 49 | — | 36 | — | 33 | 25 | 8 | — | 24 | — | 4 | 0 | — | — | 27 | — | 6 | 0 | — | |
| | | | | | — | 35 | — | 33 | 29 | 5 | — | 46 | — | 32 | 36 | 27 | — | 40 | — | 41 | 36 | 19 | |
| | | | | | — | 36 | — | 37 | 27 | 11 | — | 38 | — | 25 | 22 | 6 | — | 34 | — | 40 | 34 | 10 | |
| | 5 | O S S | — 7.7 11.0 | 2 51 35 | — | 1 | — | 1 | — | — | — | 1 | — | 0 | — | — | 1 | — | 0 | — | — | | |
| | | | | | — | 41 | — | 39 | 35 | 9 | — | 43 | — | 34 | 31 | 11 | — | 34 | — | 17 | 21 | 0 | |
| | | | | | — | 26 | — | 24 | 16 | 7 | — | 21 | — | 3 | 3 | 1 | — | 30 | — | 3 | 7 | 4 | |
| 24 | -18 | O S S | — 7.7 11.0 | 38 55 45 | 31 | 29 | 29 | 25 | 24 | 22 | 26 | 16 | 23 | 14 | 14 | 11 | 31 | 26 | 30 | 23 | 25 | 0 | |
| | | | | | 48 | 41 | 49 | 32 | 30 | 33 | 47 | 37 | 34 | 20 | 32 | 26 | 43 | 41 | 41 | 25 | 36 | 0 | |
| | | | | | 41 | 39 | 39 | 19 | 28 | 31 | 35 | 30 | 22 | 17 | 17 | 21 | 36 | 33 | 32 | 14 | 5 | | |
| | 5 | S S | 7.7 11.0 | 46 33 | 38 | 38 | 42 | 24 | 38 | 35 | 43 | 35 | 39 | 17 | 23 | 43 | 33 | 30 | 19 | 20 | 2 | — | |
| | | | | | 24 | 28 | 28 | 17 | 24 | 13 | 24 | 21 | 18 | 7 | 14 | 3 | 24 | 23 | 20 | 13 | 11 | — | |

TABLE III

EFFECT OF AGE AND STORAGE CONDITIONS ON THE SURVIVAL OF DOUGLAS FIR SEEDS IN PACKETS AT 5° C.

| Original storage | | | | Per cent germination after further storage in packets at 5° C., months | | | | | | | | | | | | | | | | | | | | | | | | |
|------------------|----------------|----------------------|--------------------|--|-----------------|----|----|----|----|-----|----|-----|---------|----|----|----|----|----|-----|----|---------------|----|----|----|----|--|--|--|
| Mos. | Temp., ° C. | Open or sealed | % Mois- ture | o | Manila envelope | | | | | | | | Tin can | | | | | | | | Foil envelope | | | | | | | |
| | | | | | 0.5 | 1 | 2 | 3 | 6 | 12 | 18 | 0.5 | 1 | 2 | 3 | 6 | 12 | 18 | 0.5 | 1 | 2 | 3 | 6 | 12 | 18 | | | |
| 6 | -18 | O | — | 81 | 79 | 82 | 78 | 59 | 57 | 25* | 82 | 88 | 86 | 76 | 70 | 73 | 38 | 83 | 83 | 86 | 79 | 59 | 64 | 37 | | | | |
| | | S | 5.8 | 83 | 91 | 85 | 71 | 62 | — | 88 | 87 | 90 | 84 | 66 | 77 | 77 | 88 | 80 | 86 | 80 | 73 | 85 | 85 | | | | | |
| | | S | 13.6 | 82 | 81 | 82 | 65 | 61 | 69 | 29* | 85 | 80 | 85 | 74 | 62 | 70 | 34 | 78 | 88 | 78 | 65 | 65 | 70 | 47 | | | | |
| 12 | 5 | O | — | 73 | 74 | 79 | 65 | 57 | 51 | 6* | 80 | 74 | 80 | 68 | 57 | 58 | 20 | 79 | 75 | 79 | 85 | 59 | 56 | 27 | | | | |
| | | S | 5.8 | 88 | 81 | 83 | 73 | 60 | 60 | — | 90 | 81 | 82 | 80 | 65 | 69 | 74 | 84 | 85 | 88 | 72 | 73 | 79 | 83 | | | | |
| | | S | 13.6 | 80 | 86 | 80 | 65 | 54 | 53 | 14* | 83 | 82 | 84 | 67 | 56 | 67 | 43 | 83 | 82 | 79 | 64 | 61 | 63 | 37 | | | | |
| 18 | -18 | O | — | 69 | 60 | 69 | 61 | 60 | 40 | 35 | 57 | 60 | 58 | 55 | 68 | 40 | 46 | 60 | 54 | 70 | 52 | 61 | 34 | 31 | | | | |
| | | S | 5.8 | 76 | 75 | 81 | 72 | 75 | 49 | 34 | 74 | 75 | 78 | 70 | 77 | 55 | — | 77 | 79 | 82 | 65 | 82 | 74 | 79 | | | | |
| | | S | 13.6 | 77 | 72 | 74 | 80 | 72 | 61 | 29 | 45 | 70 | 72 | 79 | 77 | 69 | 42 | 18 | 72 | 78 | 75 | 78 | 67 | 57 | | | | |
| 24 | 5 | O | — | 66 | 50 | 58 | 48 | 52 | 20 | 9 | 62 | 60 | 61 | 44 | 58 | 37 | 31 | 56 | 65 | 58 | 48 | 60 | 26 | 33 | | | | |
| | | S | 5.8 | 75 | 73 | 72 | 57 | 63 | 36 | 41 | 73 | 77 | 73 | 64 | 79 | 58 | — | 71 | 78 | 79 | 63 | 76 | 65 | 79 | | | | |
| | | S | 13.6 | 62 | 60 | 60 | 56 | 37 | 10 | 6 | 60 | 61 | 66 | 64 | 58 | 25 | 21 | 61 | 61 | 66 | 66 | 55 | 24 | 19 | | | | |
| 18 | -18 | O | — | 49 | — | — | — | 37 | 20 | 15 | — | 54 | — | 41 | 36 | 26 | 12 | — | 54 | — | 49 | 45 | 27 | 17 | | | | |
| | | S | 5.8 | 76 | — | — | — | 83 | 60 | 33 | 29 | — | 81 | — | 83 | 74 | 65 | 8 | 81 | — | 79 | 77 | 79 | 78 | | | | |
| | | S | 13.6 | 75 | — | — | — | 80 | 50 | 37 | 24 | — | 80 | — | 78 | 65 | 60 | 45 | 76 | — | 80 | 63 | 70 | 60 | | | | |
| 24 | 5 | O | — | 60 | — | 48 | — | 31 | 7 | 0 | — | 43 | — | 34 | 17 | 3 | 0 | — | 51 | — | 37 | 20 | 9 | 1 | | | | |
| | | S | 5.8 | 79 | — | 73 | — | 78 | 57 | 31 | 16 | 76 | — | 77 | 68 | 67 | 72 | — | 74 | — | 81 | 73 | 70 | 78 | | | | |
| | | S | 13.6 | 59 | — | 49 | — | 43 | 18 | 7 | — | 52 | — | 42 | 37 | 17 | 12 | — | 49 | — | 47 | 38 | 39 | 7 | | | | |
| 24 | -18 | O | — | 50 | 43 | 50 | 45 | 54 | 55 | — | 54 | 48 | 58 | 44 | 49 | 51 | — | 37 | 47 | 26 | 32 | 47 | 35 | — | | | | |
| | | S | 5.8 | 79 | 81 | 73 | 84 | 85 | 77 | 83 | 81 | 71 | 87 | 78 | 71 | 81 | — | 83 | 78 | 84 | 78 | 82 | 87 | — | | | | |
| | | S | 13.6 | 74 | 77 | 68 | 79 | 73 | 76 | 76 | 77 | 63 | 80 | 69 | 76 | 76 | — | 72 | 64 | 76 | 76 | 77 | 72 | — | | | | |
| 24 | 5 | O | — | 17 | 13 | 18 | 10 | 21 | 14 | — | 18 | 13 | 12 | 9 | 17 | 13 | — | 15 | 12 | 12 | 5 | 12 | 3 | — | | | | |
| | | S | 5.8 | 73 | 66 | 78 | 73 | 79 | 70 | 79 | 65 | 74 | 70 | 81 | 74 | — | 69 | 70 | 78 | 71 | 78 | 75 | — | | | | | |
| | | S | 13.6 | 42 | 38 | 28 | 42 | 35 | 52 | — | 43 | 36 | 44 | 37 | 36 | 26 | — | 48 | 30 | 41 | 36 | 34 | 24 | — | | | | |

* After 24 months in packets.

TABLE IV
EFFECT OF AGE AND STORAGE CONDITIONS ON THE SURVIVAL OF WESTERN HEMLOCK SEEDS IN PACKETS AT 5° C.

| Original storage | | | | Per cent germination after further storage in packets at 5° C., months | | | | | | | | | | | | | | | | | | |
|------------------|----------------|----------------------|---------------|--|-----------------|----|----|----|----|---------|-----|----|----|----|---------------|----|-----|----|----|----|----|----|
| Mos. | Temp., ° C. | Open or sealed | % Moisture | 0 | Manila envelope | | | | | Tin can | | | | | Foil envelope | | | | | | | |
| | | | | | 0.5 | 1 | 2 | 3 | 6 | 12 | 0.5 | 1 | 2 | 3 | 6 | 12 | 0.5 | 1 | 2 | 3 | 6 | 12 |
| 6 | -18 | O | — | 46 | 46 | 40 | 48 | 37 | 23 | 45 | 42 | 46 | 42 | 39 | 34 | 39 | 51 | 39 | 52 | 39 | 34 | |
| | | S | 7.7 | 44 | 37 | 47 | 46 | 36 | 12 | 49 | 47 | 46 | 49 | 51 | 37 | 40 | 47 | 48 | 46 | 44 | 43 | |
| | | S | 11.0 | 40 | 34 | 42 | 38 | 46 | 37 | 16 | 41 | 44 | 38 | 47 | 38 | 34 | 41 | 44 | 39 | 49 | 40 | 37 |
| 12 | 5 | O | — | 20 | 13 | 19 | 18 | 15 | 10 | 4 | 20 | 21 | 18 | 25 | 18 | 14 | 16 | 24 | 15 | 14 | 14 | 8 |
| | | S | 7.7 | 51 | 45 | 54 | 49 | 44 | 37 | 18 | 48 | 55 | 49 | 60 | 48 | 45 | 48 | 43 | 48 | 54 | 42 | 48 |
| | | S | 11.0 | 42 | 47 | 45 | 43 | 43 | 37 | 15 | 36 | 47 | 38 | 41 | 37 | 21 | 37 | 40 | 47 | 49 | 40 | 35 |
| 18 | -18 | O | — | 40 | 37 | 38 | 29 | 27 | 22 | 13 | 43 | 41 | 38 | 41 | 27 | 1 | 31 | 41 | 35 | 38 | 40 | 27 |
| | | S | 7.7 | 51 | 49 | 45 | 42 | 36 | 28 | 15 | 43 | 49 | 55 | 40 | 40 | 18 | 48 | 56 | 53 | 37 | 45 | 43 |
| | | S | 11.0 | 44 | 40 | 38 | 40 | 25 | 20 | 11 | 38 | 41 | 42 | 45 | 41 | 3 | 46 | 43 | 44 | 33 | 42 | 45 |
| 24 | 5 | O | — | 11 | 9 | 10 | 4 | 2 | 1 | 1 | 8 | 12 | 6 | 5 | 3 | 1 | 8 | 8 | 5 | 10 | 5 | 2 |
| | | S | 7.7 | 44 | 39 | 36 | 42 | 42 | 26 | 13 | 42 | 41 | 47 | 49 | 40 | 3 | 46 | 38 | 45 | 42 | 47 | 45 |
| | | S | 11.0 | 42 | 39 | 37 | 32 | 22 | 16 | 4 | 39 | 38 | 37 | 28 | 36 | 2 | 37 | 34 | 41 | 35 | 34 | 0 |
| 18 | -18 | O | — | 54 | — | 34 | — | 31 | 19 | 6 | — | 36 | — | 39 | 34 | 29 | — | 39 | — | 34 | 32 | 35 |
| | | S | 7.7 | 46 | — | 39 | — | 35 | 29 | 8 | — | 39 | — | 53 | 52 | 41 | — | 40 | — | 40 | 40 | 2 |
| | | S | 11.0 | 49 | — | 43 | — | 32 | 25 | 4 | — | 39 | — | 41 | 37 | 33 | — | 42 | — | 38 | 48 | 33 |
| 24 | 5 | O | — | 2 | — | 2 | — | 0 | — | 0 | — | 4 | — | 2 | — | 1 | — | 3 | — | 0 | — | 0 |
| | | S | 7.7 | 51 | — | 39 | — | 36 | 26 | 6 | — | 48 | — | 47 | 41 | 39 | — | 43 | — | 35 | 44 | 35 |
| | | S | 11.0 | 35 | — | 28 | — | 17 | 9 | 1 | — | 30 | — | 25 | 23 | 26 | — | 30 | — | 23 | 28 | 24 |
| 24 | -18 | O | — | 38 | 32 | 47 | 34 | 25 | 33 | 37 | 37 | 42 | 40 | 27 | 31 | 28 | 31 | 37 | 31 | 26 | 41 | 30 |
| | | S | 7.7 | 55 | 50 | 51 | 41 | 35 | 40 | 43 | 45 | 48 | 48 | 32 | 43 | 45 | 52 | 46 | 47 | 37 | 42 | 47 |
| | | S | 11.0 | 45 | 28 | 44 | 46 | 32 | 36 | 35 | 39 | 38 | 34 | 33 | 38 | 32 | 39 | 45 | 30 | 28 | 36 | 38 |
| 24 | 5 | O | — | 46 | 34 | 42 | 34 | 26 | 43 | 38 | 51 | 45 | 44 | 29 | 41 | 46 | 44 | 53 | 36 | 31 | 40 | 43 |
| | | S | 7.7 | 40 | 35 | 35 | 27 | 21 | 28 | 22 | 29 | 34 | 34 | 18 | 32 | 28 | 36 | 32 | 25 | 21 | 32 | 22 |
| | | S | 11.0 | 33 | 40 | 35 | 27 | 21 | 28 | 22 | 29 | 34 | 34 | 18 | 32 | 28 | 36 | 32 | 25 | 21 | 32 | 22 |

Germination tests made after 24 months in the packets, however, again revealed the advantage of sealed storage of dry seeds (5.8 per cent moisture) for maintenance of viability in packets. The same general effects of packeting were noted after original storage at 5° C.

Some of the effects of sealed storage of Douglas fir seeds for 12 months upon subsequent deterioration in tin cans at 5° C. are pictured in Figure 3 B. A comparison of A and B of Figure 3 emphasizes the different rates of viability loss in tin cans at 5° and 30° C.

The germination of seeds of Western hemlock packeted and held at 5° C. is shown in Table IV. In general, the effects are the same as those described for Douglas fir.

Aluminum foil packets. Previous work (3) showed vinyl-laminated aluminum foil protected onion seeds of low moisture content from rapid deterioration in a humid atmosphere at 30° C. To extend our knowledge of the efficacy of this material for seed packeting, it was used for comparison with Manila envelopes and tin cans in the present experiment. The results are shown in Tables I, II, III, and IV. The foil was entirely satisfactory for seeds of all moisture contents when the packets were stored at 5° C. However, at the higher temperature of 30° C. when the moisture contents of the seeds were high, the foil brought about a very rapid deterioration (Tables I and II). A comparison with tin cans revealed that the harmful effect was greater than that imposed by the moisture content of the seeds. There is a possibility that some toxic volatile substance is present and is released under conditions of high temperature and high humidity. Some preliminary tests in which vinyl-laminated aluminum foil has been compared with foil with no coating indicate that this may be the case. Certainly, all of these facts should be taken into consideration before aluminum foil is used extensively for seed packeting.

DISCUSSION

These tests have demonstrated once again that subfreezing is to be preferred over above-freezing temperature for the maintenance of high quality conifer seeds. It has been shown further that the better the original storage condition, the higher the value of the seeds when they are removed from storage for planting or packeting or shipment. This invalidates objections to the use of "frozen" seed. Experimental results have shown that Douglas fir and Western hemlock seeds stored at -18° C. for as long as two years retain their full germination capacity and do not suffer, by virtue of their storage, upon transfer to a higher temperature. On the contrary, they are more resistant to subsequent deterioration than seeds which have been stored above freezing where they have lost some of their germination vigor. For example, Douglas fir seeds packeted in Manila envelopes held at 30° C. after six months of original open storage deteriorated at the

same rate whether the original storage temperature was -18° or 5° C. The same relationship held after 12 months of original storage though the actual germination percentages were somewhat reduced. After 24 months of original storage, however, the great decrease in germination which took place during storage at 5° C. made the seeds worthless for packeting, while those stored originally at -18° C. were still of high quality. In other words, any condition which prevents deterioration of the seeds in storage, whether that condition be subfreezing temperature, reduced moisture content, or both, permits a greater delay in planting. Some seeds held at a temperature as low as 5° C., but in high humidity, are worthless after a few months, while seeds of the same lots stored at subfreezing temperatures for years may be as good as fresh seeds in every way.

Six months of original storage at -18° C. plus 12 months in packets or 12 months of original storage at -18° C. plus 6 months in packets had the same effect on subsequent germination of Douglas fir seeds when the packets were stored at 5° C. Furthermore, the conditions were as favorable for retention of viability as an original storage period of 18 months at -18° C. When the packets were stored at 30° C., however, there was a greater retention of germination capacity after 12 months of original storage at -18° C. plus 6 months in packets, than after 6 months of original storage at -18° C. plus 12 months in packets. The survival of seeds upon removal from original storage and packeting, then, is directly related to the time they are exposed to an unfavorable temperature or humidity, whether that time be during the original storage period or after packeting for sale or shipment.

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COMPARATIVE EFFECT OF FUNGICIDES ON OXYGEN UPTAKE AND GERMINATION OF SPORES¹

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SUMMARY

The effect of a number of water soluble and relatively insoluble fungicides on the oxygen uptake and germination of spores of *Neurospora sitophila*, *Monilinia fructicola*, *Myrothecium verrucaria*, *Cephalosporium acremonium*, *Alternaria oleracea*, *Aspergillus niger*, *Stemphylium sarcinaeforme*, and *Rhizopus nigricans* was studied. These species varied widely in physiological activity as indicated by oxygen uptake in presence of 1 per cent sucrose. The species are listed in descending order of activity. The ratio of exogenous to endogenous rate was constant for a given species.

Varying concentrations of water soluble fungicides (or wettable fungicides) were added to 10 mg. of spores in suspension and oxygen uptake determined over a 5-hour period, after which the spores were tested for germination. Difficultly soluble fungicides were dissolved in 2 per cent acetone and a dosage series obtained by varying the volume. Spores were pretreated in such solutions for 15 minutes, then oxygen uptake determined.

Oxygen uptake-time curves in the presence of fungicides were of three types: a depression increasing with concentration, a depression followed by an increase in uptake, and no appreciable effect with concentration. These effects were summarized by means of dosage-response curves based on per cent of control, and the ED₅₀ values in micrograms of fungicide per milligram of spores determined.

In general, the ED₅₀ values were lower for germination than for oxygen uptake, indicating that germination was a more sensitive measure of toxicity than oxygen uptake. *Monilinia fructicola* as a rule was the most sensitive species in both responses and *Aspergillus niger* the least sensitive. Silver nitrate, copper sulfate, and mercuric chloride markedly retarded oxygen uptake and reduced germination for all species. Other compounds such as cadmium chloride, cycloheximide and dichlone were toxic to spore germination but did not reduce oxygen uptake for some species. Chloranil, 2-heptadacyl-2-imidazoline, and ferbam tended to give responses of comparable magnitude for oxygen uptake and germination for most species, while for captan these varied widely. Nabam was without effect.

Marked increases in oxygen uptake were observed, particularly for *Neurospora sitophila* with most fungicides. Wettable fungicides tended to have less effect on oxygen uptake than comparable ones dissolved in dilute acetone, but there was little difference in germination response. In general, the dosage-response curves were flatter for oxygen uptake than for germination.

INTRODUCTION

While there is a considerable amount of information on the effect of fungicides on the germination of fungus spores, there is much less known regarding their effects on respiration. Accordingly, a study has been made on

¹ These investigations were conducted in cooperation with the United States Atomic Energy Commission, Contract AT(30-1)-788. A preliminary report was presented before the American Phytopathological Society, Estes Park, Colorado, August 1954 (11).

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the comparative effect of fungicides on oxygen uptake and germination of fungus spores. It was considered of especial interest to contrast the relative sensitivity of oxygen uptake and germination as measures of toxicity, and also to demonstrate any patterns of oxygen uptake which might throw some light on the nature of fungicidal action. Included in these studies were the spores of eight different species of fungi and some 20 different chemicals, in the course of which over 1000 different oxygen uptake curves have been obtained.

MATERIALS AND METHODS

FUNGI

Conidia of the following fungi were studied most extensively: *Neurospora sitophila* (Mont.) Shear & Dodge, *Monilinia fructicola* (Wint.) Honey, *Alternaria oleracea* Milbraith, *Aspergillus niger* van Tiegh (Strain TC-215-4247)², and *Myrothecium verrucaria* (Alb. & Schw.) Ditm. ex Fr. (Strain 1334.2)². Spores were used to a lesser extent from cultures of *Rhizopus nigricans* Ehr., *Cephalosporium acremonium* Corda, and *Stemphylium sarcinaeforme* (Cav.) Wilts. All fungi were cultured and the spores harvested as reported previously (19). *Myrothecium verrucaria* was cultured on potato dextrose agar slants in large tubes. All spores were used from cultures about one to three weeks old, usually one to two weeks, except those of *Monilinia fructicola* which were from cultures six to nine days old. Within these age limits there were no significant differences in response of the different species.

The quantity of spores used was expressed on a fresh weight basis using the spore measurements reported earlier (19). A fresh culture of *Rhizopus nigricans* (+ strain) with normal-sized spores was obtained². The mean diameters from 100 spores were $8.81 \times 7.89 \mu$. The mean volume as a prolate sphaeroid weighted for frequency distribution was 322.54 cu. μ , which with a specific gravity of 1.1 would give a calculated weight for 1,000,000 spores of 0.355 mg. Likewise, spores of *Myrothecium verrucaria*, not measured before, had mean diameters of $7.03 \times 2.69 \mu$ and a mean volume as a prolate sphaeroid of 26.57 cu. μ . One million spores at specific gravity of 1.1 would weigh 0.029 mg.

Germination tests were made at the end of the oxygen uptake test, that is after five or six hours, to determine viability. A sample of spores was taken from the manometric flask, diluted with distilled water, and the appropriate nutrient added to give a final concentration of about 100,000 spores per ml. The spores in drops were placed on glass slides to germinate in the usual manner (1). Counts were made on the germination of duplicate

² The cultures of *Aspergillus niger* and *Myrothecium verrucaria* were kindly furnished by Dr. P. B. Marsh, Plant Industry Station, Beltsville, Maryland, and *Rhizopus nigricans* by Dr. W. J. Robbins, New York Botanical Garden, New York, New York.

samples of 100 spores from each flask. Tests conducted with spores of *Neurospora sitophila* and *Monilinia fructicola* indicated that insufficient chemical was carried over from the flask to affect the germination since washing and centrifuging the aliquot of spores did not increase the percentage of germination. Nutrients and germination temperatures have been reported earlier (19). Spores of *Myrothecium verrucaria* were germinated in sucrose and yeast extract with phosphate buffer, according to the method of Mandels and Darby (15), at 21° C. for 20 hours. The spores of *Myrothecium verrucaria* and *Monilinia fructicola* were found to have germinated partially in the less toxic solutions in the manometric flasks when the samples were taken. This preliminary measure of germination was inadequate for expressing final results.

FUNGICIDES

The fungicides studied comprised both water soluble and relatively insoluble inorganic and organic compounds. The soluble inorganic compounds were mostly salts of heavy metals. These comprised copper sulfate, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; silver nitrate, AgNO_3 ; zinc chloride, ZnCl_2 ; cadmium chloride, $\text{CdCl}_2 \cdot 2\frac{1}{2}\text{H}_2\text{O}$; mercuric chloride, HgCl_2 ; cerous sulfate, $\text{Ce}_2(\text{SO}_4)_3 \cdot 8\text{H}_2\text{O}$; sodium arsenate, $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$, and lime sulfur (expressed as calcium polysulfides). All results with the salts of the heavy metals and cerium have been expressed on the basis of the cation. Orientation tests involving changes in technique, were largely made with copper sulfate.

The soluble organic chemicals were phenol; nabam, disodium ethylenebis(dithiocarbamate); malachite green; and cycloheximide (Acti-dione³), 3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide.

The relatively water insoluble chemicals were comparatively pure and unformulated preparations of some of the common and more effective fungicides,³ namely, ferbam, ferric dimethyldithiocarbamate; the free base of glyodin, 2-heptadecyl-2-imidazoline; chloranil, tetrachloro-*p*-benzoquinone; dichlone, 2,3-dichloro-1,4-naphthoquinone; and captan, *N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide. These materials were all studied in solution in 2 per cent acetone. In addition to these, several commercial formulations were tested in suspension, in particular Magnetic 95 wettable sulfur (Stauffer) and to a limited extent several other sulfur preparations, as well as Crag 341B (18 per cent 2-heptadecyl-2-imidazoline, 10 per cent 2-pentadecyl-2-imidazoline, <1 per cent 2-heptadecenyl-2-

³ These purified fungicides were generously supplied as follows: ferbam, E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.; the free base of glyodin, Carbide and Carbon Chemicals Co., New York, N. Y.; chloranil and dichlone, Naugatuck Chemical Division, Naugatuck, Conn.; and captan, Stauffer Chemical Co., New York, N. Y. The Acti-dione was kindly furnished by the Upjohn Co., Kalamazoo, Mich.

imidazoline, 3.3 per cent 1-hydroxyethyl-2-heptadecyl-2-imidazoline), Fermate (70 per cent ferbam), Tersan [50 per cent thiram or bis(dimethylthiocarbamoyl) disulfide], wettable Spergon (50 per cent chloranil), and wettable Phygon (97 per cent dichlone).

All chemicals were studied in a concentration series. The dose ratio in most cases was the $\sqrt{10}$, while a ratio of 5 was used with Magnetic 95 sulfur. Usually a series of seven concentrations was employed, thus with the dose ratio of $\sqrt{10}$ a thousand-fold range was covered. This was considered necessary in order to demonstrate both inhibition and stimulation, if present.

TECHNIQUES

An 18-unit rotary Warburg apparatus set at 30° C. was used in these studies. The shaking speed for the rotary apparatus was 112 oscillations per minute for most of the studies; this was later reduced to 75. In general, the operating procedures outlined by Umbreit *et al.* (25) were followed. However, it was found that if the first step was to grease the rim of the center well and add the KOH solution, contaminations from the creeping of KOH were eliminated. Flasks were calibrated by the method of Loomis (8). Drying and storage of the flasks and plugs were facilitated by use of a wooden frame with numbered circular holes appropriate for holding the inverted flasks and plugs. In general, for orthodox tests 0.2 ml. of 10 or 20 per cent KOH was added to the center well, and 0.2 ml. of fungicide chemical was placed in the sidearm. In the body of the flask there were placed 0.8 ml. of nutrient (usually 2.5 per cent of sucrose which gave a final concentration of 1 per cent) and 1 ml. of spore suspension (usually 10 mg. fresh weight). Finally 2 cm. squares of filter paper, folded accordion-wise, were placed in the center well. In most cases all concentrations of chemical or other variables were run in duplicate. There were also duplicate thermobarometers. Readings were taken every half hour and occasionally every quarter hour. One hour after starting, the sidearms were tipped to introduce the fungicide, and readings on oxygen uptake continued for five hours.

Spores were pretreated with the relatively insoluble or unstable chemicals, i. e., chemicals which broke down with the release of gases. In the former case the chemical was dissolved in acetone and the solution added to water to give a final concentration of 2 per cent acetone. Repeated tests have shown that 2 per cent acetone does not affect oxygen uptake or germination. One ml. of spore suspension (usually 10 mg. fresh weight) was added to a series of volumes of the dilute acetone solution ranging in steps of $\sqrt{10}$ from 1 ml. to 1000 ml., and kept stirred for 15 minutes. The spores were then removed by centrifuging and washed once, made up to 1 ml. and added to the body of the manometric flasks. Oxygen uptake on the

pretreated spores was determined for a total period of five hours. In the latter case of unstable compounds, specifically lime sulfur and nabam, constant volumes of 10 ml. and concentrations varying by $\sqrt{10}$ were used. Here again the spores were exposed for 15 minutes and removed by centrifuging and washed. With lime sulfur care was taken not to use solutions so dilute that cloudiness from products of hydrolysis showed in the 15-minute period.

RESULTS

It is customary to give the results of oxygen uptake studies in the form of curves. However, since over 1000 curves were obtained, excluding duplicates, in the present studies, only in special cases are the curves shown. For most of the data only the total microliters of oxygen taken up over the 5-hour period following addition of fungicide have been reported for the varying amounts of chemical and spores. Furthermore, many of these data have been condensed into a single value, the ED₅₀; that is, the effective dose or quantity required to reduce the oxygen uptake to 50 per cent of the controls. Germination results are also given in the customary ED₅₀ values (2). Both of these ED₅₀ values were obtained by interpolation from straight lines or smooth curves drawn on logarithmic-probability paper in the usual manner (13). The authors have shown in earlier papers the importance of total amount of chemical to total spores (20, 21). In these studies only the external amount of chemical is known, nevertheless it is believed to be important to express the ratio rather than the concentration. Hence, all values are given as ratios, usually as micrograms per milligram of spores.

EFFECT OF NUTRIENTS

The oxygen uptake of fungus spores may be determined without an external source of energy, i.e., endogenous uptake, or by adding nutrients to the media, i. e., exogenous uptake. For many of the spores studied the endogenous rate is so low that it was considered essential to make determinations of exogenous oxygen uptake. A comparison of the two rates for the spores of the eight species in two separate tests each is shown in Table I. The nutrient was sucrose in 1 per cent solution or a total of 2 mg. and in most cases there were 10 mg. of spores. It will be seen that the exogenous oxygen uptake ranges from about 1 to 22 times the endogenous. The relatively close agreement between the replicates for most species indicates a fairly constant ratio for the species and thus a significant difference between species, while even more constant is the ratio of exogenous to endogenous oxygen uptake. In the presence of sucrose there is about a 10-fold range in oxygen uptake from the most active, *Neurospora sitophila*, to the least active, *Rhizopus nigricans*.

Sucrose has been used as a source of energy by Yoder (27) and others;

TABLE I
COMPARISON OF ENDOGENOUS AND EXOGENOUS OXYGEN UPTAKE FOR
SPORES OF 8 SPECIES OF FUNGI

| Fungus | μ l. O ₂ taken up in 5 hours per mg. spores in 2 tests | | |
|----------------------------------|--|--------------------------|-------------------------|
| | Endogenous | Exogenous, 1% sucrose | Exogenous Endogenous |
| <i>Neurospora sitophila</i> | 8.6 6.7 | 68.6 41.2 | 8.0 6.1 |
| <i>Monilinia fructicola</i> | 5.1 4.5 | 54.2 47.2 | 10.6 10.5 |
| <i>Cephalosporium acremonium</i> | 2.7 1.7 | 42.8 37.1 | 15.8 21.9 |
| <i>Myrothecium verrucaria</i> | 8.8 16.1 | 22.1 40.0 | 2.5 2.5 |
| <i>Stemphylium sarcinaeforme</i> | 2.0 1.3 | 21.7 14.2 | 10.8 11.1 |
| <i>Alternaria oleracea</i> | 7.1 8.2 | 17.4 17.7 | 2.5 2.2 |
| <i>Aspergillus niger</i> | 12.8 6.3 | 17.7 9.7 | 1.4 1.5 |
| <i>Rhizopus nigricans</i> | 8.5 4.7 | 9.2 4.3 | 1.1 0.9 |

glucose has also been commonly used. A comparison of the two sugars alone (1 per cent), and in the presence of two levels of copper sulfate, on oxygen consumption and subsequent germination was made on the spores of *Neurospora sitophila*, and the results are given in Table II. There is no significant difference between effects of glucose and sucrose on oxygen uptake or germination either in the absence or presence of the toxicant copper. It was decided to use sucrose throughout in these studies.

Since sucrose was adopted as the nutrient, it was considered desirable to determine the effect of different amounts in the presence of different quantities of a toxicant. The results with spores of *Neurospora sitophila* and *Monilinia fructicola* and copper sulfate are presented in Table III. A repetition gave similar results. It will be seen that as expected the oxygen uptake is greatest at the highest amount of sucrose and, further, the differences in response at the different levels of copper are increased. It also appears that the germination of the spores remains the same or possibly is decreased at the high sugar concentration. Thus the high concentration of sucrose provides a more sensitive medium for studying the effects of fungicides on the oxygen uptake and germination of spores. Accordingly, this

TABLE II
EFFECT OF GLUCOSE AND SUCROSE ON OXYGEN UPTAKE AND GERMINATION OF
SPORES OF *NEUROSPORA SITOPHILA* IN PRESENCE OF COPPER SULFATE

| Nutrient added, 1% | Oxygen uptake (μl. per mg. spores in 5 hrs.) and germination (%) in presence of various amounts of Cu (μg. per mg. spores) | | | | | |
|-----------------------|--|-------|--------------------|-------|--------------------|-------|
| | 0 | | 0.063 | | 2.0 | |
| | μl. O ₂ | Germ. | μl. O ₂ | Germ. | μl. O ₂ | Germ. |
| None | 5.8 | 94 | 6.5 | 90 | 13.5 | 41 |
| Glucose | 36.0 | 95 | 40.5 | 51 | 21.9 | 22 |
| Sucrose | 37.0 | 95 | 42.8 | 51 | 25.7 | 17 |

high concentration of sucrose has been used as the standard. This amount, 0.2 mg. per milligram of spores, is equivalent to 1 per cent in a 2-ml. solution containing 10 mg. of spores.

It was considered of interest to determine the effect of more complex nutrient media than sucrose, namely, the nutrients which are used as germination media for *Monilinia fructicola*, *Neurospora sitophila*, and *Myrothecium verrucaria*. In the case of *M. fructicola* a test was made of 1 per cent glucose compared to 1 per cent glucose plus 0.225 per cent magnesium sulfate [Lin's solution (7)] plus 1 per cent ammonium sulfate. Three of the ingredients of the modified Fries solution (22) were tested on *N. sitophila*, namely, 1 per cent sucrose contrasted with 1 per cent sucrose plus 1 per cent ammonium sulfate plus biotin at 4 micrograms per liter. The tests with *M. verrucaria* consisted of a comparison of 1 per cent sucrose against 1 per cent sucrose plus 1 per cent yeast extract in phosphate buffer

TABLE III
EFFECT OF VARYING AMOUNTS OF SUCROSE AND COPPER SULFATE ON OXYGEN
UPTAKE AND GERMINATION OF SPORES OF *NEUROSPORA SITOPHILA*
AND *MONILINIA FRUCTICOLA*

| Fungus | Sucrose, mg. per mg. spores | Oxygen uptake (μl. per mg. spores in 5 hrs.) and germination (%) in presence of various concentrations of Cu (μg. per mg. spores) | | | | | | | |
|-----------------------------|--------------------------------------|---|-------|--------------------|-------|--------------------|-------|--------------------|-------|
| | | 0 | | 0.063 | | 0.63 | | 6.3 | |
| | | μl. O ₂ | Germ. | μl. O ₂ | Germ. | μl. O ₂ | Germ. | μl. O ₂ | Germ. |
| <i>Neurospora sitophila</i> | 0 | 5.3 | 96 | 6.1 | 96 | 11.9 | 50 | 4.3 | 4 |
| | 0.002 | 8.3 | 95 | 7.7 | 96 | 16.9 | 72 | 5.3 | 7 |
| | 0.02 | 22.1 | 96 | 26.5 | 82 | 26.7 | 41 | 3.1 | 2 |
| | 0.2 | 26.0 | 96 | 41.3 | 90 | 30.7 | 30 | 6.2 | 3 |
| <i>Monilinia fructicola</i> | 0 | 5.1 | 95 | — | — | 6.8 | 89 | 6.7 | 16 |
| | 0.002 | 6.1 | 98 | 8.6 | 98 | 9.6 | 84 | 7.4 | 13 |
| | 0.02 | 42.5 | 98 | — | — | 20.6 | 52 | 9.8 | 2 |
| | 0.2 | 54.2 | 96 | 54.3 | 98 | 33.4 | 72 | 17.3 | 7 |

according to Mandels and Darby (15). Also all comparisons were made at 1 or 3 levels of copper sulfate and in one instance 3 levels of silver nitrate.

The results with *Monilinia fructicola* are shown for copper sulfate and silver nitrate in Table IV. In both cases the oxygen uptake of the control was increased about 50 per cent by the addition of the ammonium sulfate and magnesium sulfate to the dextrose, while in the presence of copper or silver, oxygen uptake was increased considerably more. Germination responses were less marked. In a third test the mixture, glucose, ammonium sulfate, and magnesium sulfate, was compared to the mixture without the magnesium sulfate using copper sulfate as the toxicant. The oxygen up-

TABLE IV

EFFECT OF GLUCOSE AND A MIXTURE OF GLUCOSE, AMMONIUM SULFATE AND MAGNESIUM SULFATE ON OXYGEN UPTAKE AND GERMINATION OF SPORES OF *MONILINIA FRUCTICOLA* IN PRESENCE OF COPPER SULFATE AND OF SILVER NITRATE

| Toxicant | Nutrient added, % | Oxygen uptake (μ l. per mg. spores in 5 hrs.) and germination (%) in presence of various concentrations of toxicant (μ g. per mg. spores) | | | | | | | |
|----------|--|---|-------|-------------------------|-------|-------------------------|-------|-------------------------|-------|
| | | 0 | | 0.20 | | 0.63 | | 2.0 | |
| | | μ l. O ₂ | Germ. | μ l. O ₂ | Germ. | μ l. O ₂ | Germ. | μ l. O ₂ | Germ. |
| Cu | Glucose, 1 | 44.8 | 97 | 35.0 | 97 | 26.1 | 79 | 15.9 | 20 |
| | Glucose, 1 (NH ₄) ₂ SO ₄ , 1 MgSO ₄ , 0.225 | 68.7 | 93 | 65.4 | 95 | 68.2 | 94 | 52.2 | 71 |
| | | | | | | | | | |
| Ag | Glucose, 1 | 45.4 | 94 | 14.7 | 25 | 13.8 | 26 | 4.8 | 2 |
| | Glucose, 1 (NH ₄) ₂ SO ₄ , 1 MgSO ₄ , 0.225 | 66.7 | 93 | 45.1 | 73 | 26.7 | 35 | 11.8 | 5 |
| | | | | | | | | | |

take and germination in the absence of magnesium sulfate was at least equal to that in its presence, thus indicating that magnesium sulfate had not contributed to the activity of the mixture. In general, similar results were obtained in the tests with *Neurospora sitophila*. However, here, the addition of ammonium sulfate and biotin to sucrose more than doubled the oxygen uptake over that with sucrose alone, and the differences were even more marked in the presence of high concentrations of copper. Again the effect on germination was less pronounced. A separate test indicated that the biotin did not contribute appreciably to the mixture of sucrose and ammonium sulfate. The results with *Myrothecium verrucaria* are given in Table V. The addition of yeast extract to the sucrose increased the oxygen uptake of the control 11-fold and the difference was magnified with increasing amounts of copper. Also there was no reduction of germination in the presence of copper.

TABLE V

EFFECT OF SUCROSE AND A MIXTURE OF SUCROSE AND YEAST EXTRACT IN PHOSPHATE BUFFER ON OXYGEN UPTAKE AND GERMINATION OF SPORES OF MYROTHECIUM VERRUCARIA IN PRESENCE OF COPPER SULFATE

| Nutrient added, 1 % | Oxygen uptake (μl. per mg. spores in 5 hrs.) and germination (%) in presence of various concentrations of Cu (μg. per mg. spores) | | | | | | | |
|------------------------|---|-------|--------------------|-------|--------------------|-------|--------------------|-------|
| | 0 | | 0.63 | | 2.00 | | 6.3 | |
| | μl. O ₂ | Germ. | μl. O ₂ | Germ. | μl. O ₂ | Germ. | μl. O ₂ | Germ. |
| Sucrose | 4.6 | 95 | 3.2 | 96 | 2.8 | 34 | 1.7 | 7 |
| Sucrose, yeast extract | 50.4 | 95 | 47.8 | 95 | 47.5 | 93 | 46.9 | 93 |

WATER SOLUBLE COMPOUNDS

Oxygen Uptake Curves

Some representative curves for oxygen uptake are shown in Figures 1 and 2. The results are plotted as microliters of oxygen taken up per milligram of fresh spore weight for each successive hour for a total of six hours. Initial rates, i.e., for the first hour, for the control spores for the various species have been summarized from the available data with means and standard deviations as follows:

| | | | |
|----------------------------------|------------|----------------------------------|-----------|
| <i>Neurospora sitophila</i> | 12.5 ± 4.0 | <i>Alternaria oleracea</i> | 3.9 ± 1.6 |
| <i>Monilinia fructicola</i> | 9.9 ± 1.8 | <i>Aspergillus niger</i> | 3.2 ± 0.3 |
| <i>Myrothecium verrucaria</i> | 9.4 ± 2.1 | <i>Stemphylium sarcinaeforme</i> | 2.9 ± 0.3 |
| <i>Cephalosporium acremonium</i> | 7.9 ± 0.3 | <i>Rhizopus nigricans</i> | 1.4 ± 0.2 |

There is a 9-fold difference in respiration between the most active, *N. sitophila*, and the least active, *R. nigricans*. However, the rate falls off rapidly for *N. sitophila* so that by the sixth hour it is usually only half or less than half of the original rate. In contrast the oxygen uptake for *A. niger* falls relatively slowly. The curves for *A. oleracea* and *M. verrucaria* are intermediate. *Monilinia fructicola* is different in that after an early drop it usually rises to a maximum during the fourth hour and then falls off so that the rate during the sixth hour is about the same as the initial rate. *Cephalosporium acremonium* from the beginning rises to a maximum between the first and second hour, then drops off. The oxygen uptake curves for *Stemphylium sarcinaeforme* tend to rise slowly during the six-hour period, while those of *Rhizopus nigricans* fall off.

The responses to toxicants appear to be of three general types. The most common is a depression in oxygen uptake increasing with concentration as shown in Figure 1 for the action of mercuric chloride on the spores of three organisms. The second type of response is an increase in oxygen uptake at lower or intermediate concentrations of toxicant. This increase is

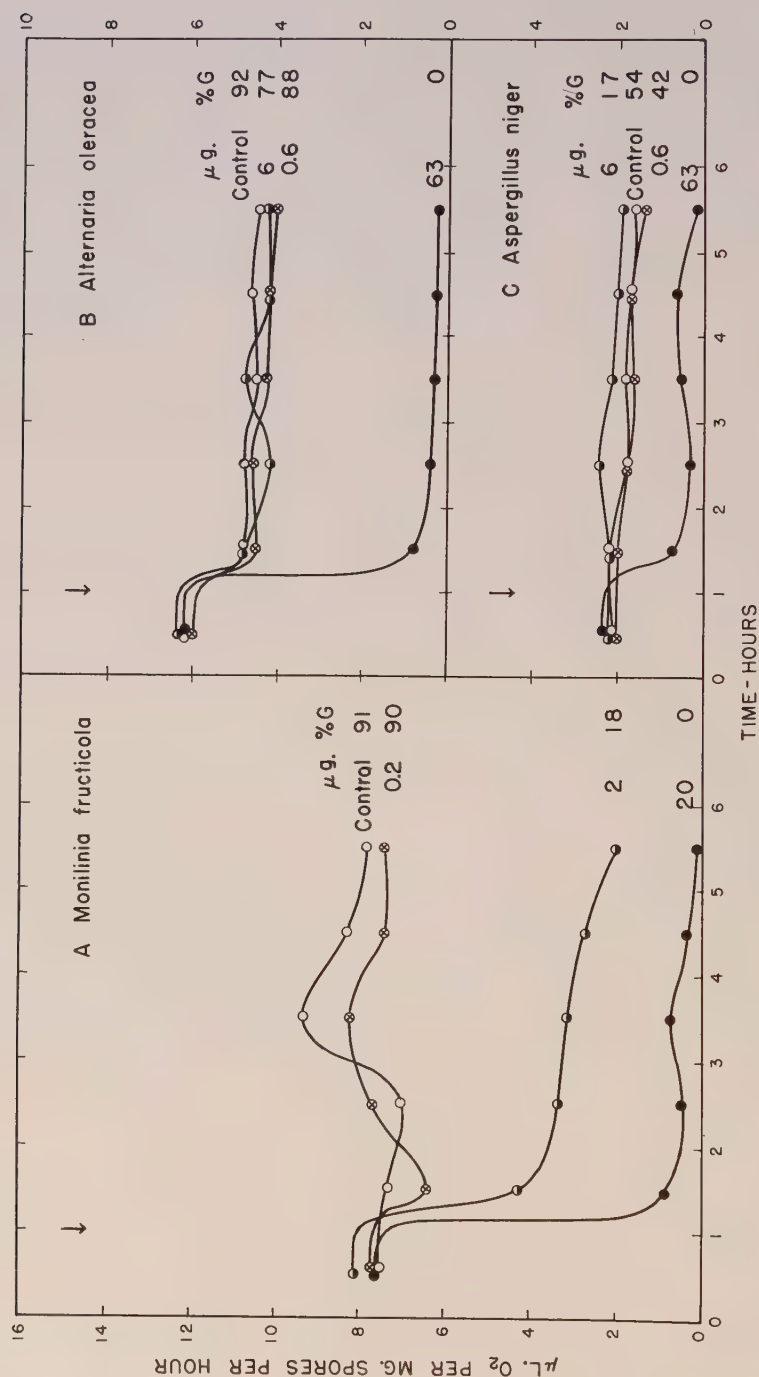


FIGURE 1. Oxygen uptake curves and germination results 5 hours after adding toxicant. Effect of mercuric chloride on (A) *Monilinia fruticicola*, (B) *Alternaria oleracea*, and (C) *Aspergillus niger*. Toxicant added as indicated by arrow at end of first hour.

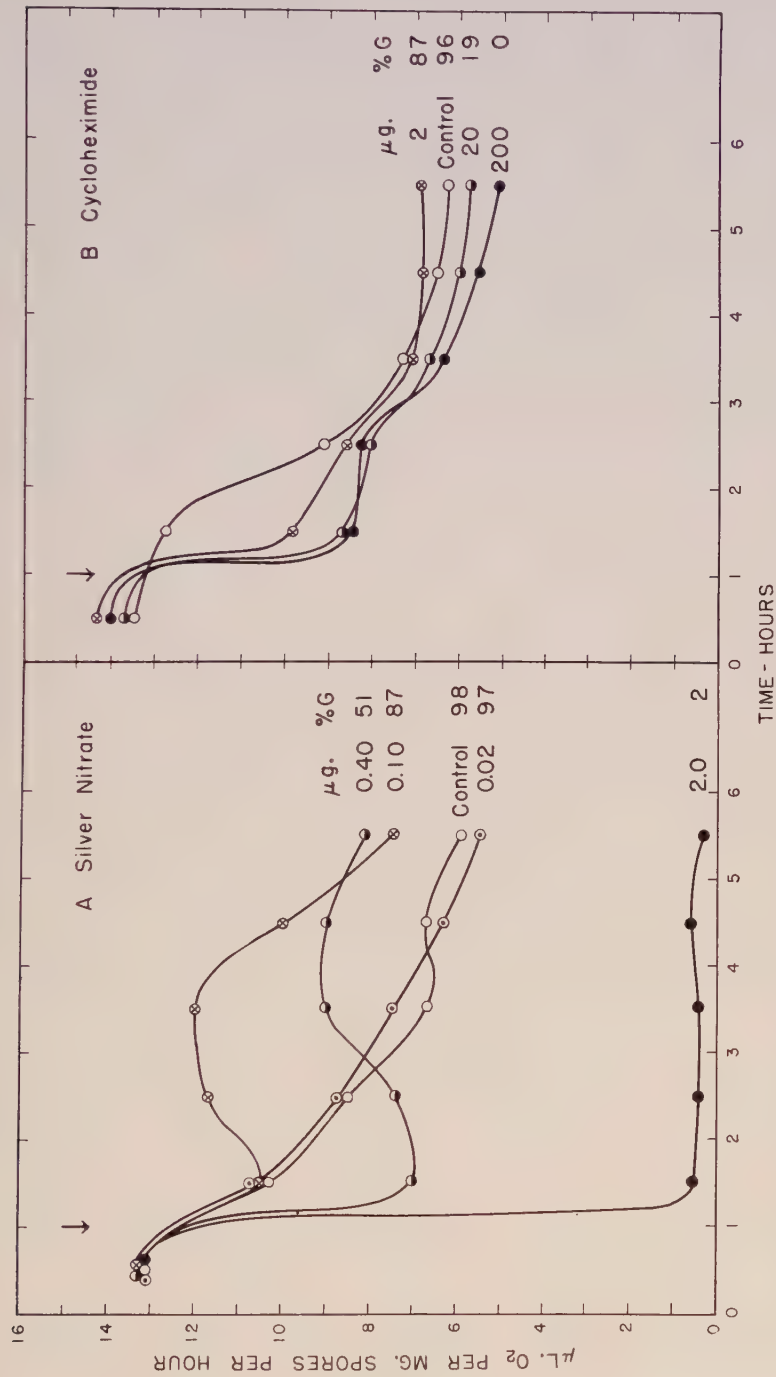


FIGURE 2. Oxygen uptake curves and germination results 5 hours after adding toxicant. Response of *Neurospora sitophila* to (A) silver nitrate and (B) cycloheximide. Toxicant added as indicated by arrow at end of first hour.

usually preceded by a depression as is shown in Figure 2 A for silver nitrate on *Neurospora sitophila*. In the responsive range increasing concentrations of toxicant produce greater initial depression and less subsequent stimulation. In the third type there is little or no response to increasing concentrations as is illustrated in Figure 2 B for cycloheximide on *N. sitophila*. The extreme case of this results in curves at all concentrations blending into the control curve.

ED₅₀ Values

The effect of chemical concentration on repression of oxygen uptake may be shown by means of dosage-response curves in which the uptake expressed as per cent of control is plotted against the concentration on logarithmic-probability paper as is commonly done for inhibition of germination. Examples from the curves of Figure 1 A and Figure 2 B are shown respectively in Figure 3 A and B, for both oxygen uptake and germination. In order to simplify the data of Figures 1 and 2, intermediate curves have been omitted; however, the points derived from these intermediate values are shown in Figure 3. By means of such dosage-response curves ED₅₀ values may be obtained. These values for a number of water soluble compounds and five fungi are summarized in Tables VI and VII. It will be seen that with one exception the ED₅₀ values are lower for germination than for oxygen uptake. That is, germination of fungus spores is a more sensitive measure of toxic response than is oxygen uptake. Only with cycloheximide on *M. fructicola* was the oxygen uptake higher. *Neurospora sitophila* and *Monilinia fructicola* are the most sensitive fungi tested and *Aspergillus niger* the most resistant. The resistance of *A. niger* is doubtless correlated with its low physiological activity as indicated by its low rate of oxygen uptake. Silver is the most effective toxicant to all fungi studied,

TABLE VI

EFFECTIVENESS OF VARIOUS WATER SOLUBLE TOXICANTS IN RETARDING OXYGEN UPTAKE AND GERMINATION OF SPORES OF *NEUROSPORA SITOPHILA* AND *MONILINIA FRUCTICOLA*

| Toxicant | ED ₅₀ values in μ g. per mg. of spores | | | |
|-----------------|---|-------------|-----------------------------|-------------|
| | <i>Neurospora sitophila</i> | | <i>Monilinia fructicola</i> | |
| | O ₂ uptake | Germination | O ₂ uptake | Germination |
| Silver | 0.66 | 0.40 | 0.30 | 0.14 |
| Copper | 1.31 | 0.40 | 2.14 | 1.65 |
| Mercury | 1.80 | 1.16 | 1.60 | 1.20 |
| Cadmium | > 1124 | 2.6 | 1.12 | 0.56 |
| Zinc | > 654 | > 654 | 470 | 54 |
| Cerium | > 200 | > 200 | 40 | 5.2 |
| Sodium arsenate | > 6320 | 740 | 20 | 1.4 |
| Phenol | 50 | 40 | > 200 | > 200 |
| Malachite green | 9.2 | 3.7 | 13 | 2.4 |
| Cycloheximide | > 200 | 92 | 2.6 | 8.4 |

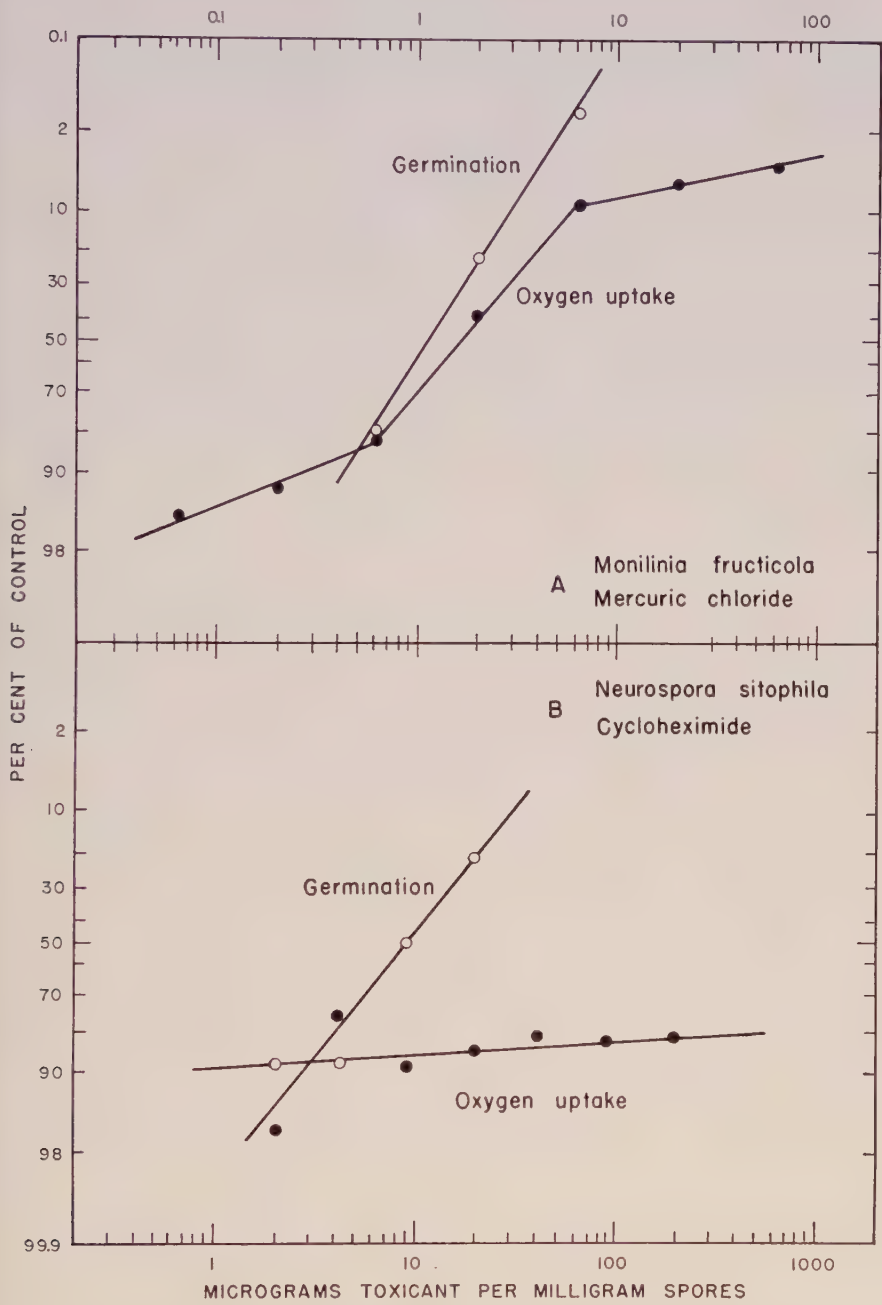


FIGURE 3. Comparison of dosage-response curves for oxygen uptake and germination. (A) Mercuric chloride on *Monilinia fructicola* derived from Figure 1 A. (B) Cycloheximide on *Neurospora sitophila* derived from Figure 2 B.

TABLE VII

EFFECTIVENESS OF VARIOUS WATER SOLUBLE TOXICANTS IN RETARDING OXYGEN UPTAKE AND GERMINATION OF SPORES OF *MYROTHECIUM VERRUCARIA*, *ALTERNARIA OLERACEA*, AND *ASPERGILLUS NIGER*

| Toxicant | ED ₅₀ values in $\mu\text{g. per mg. of spores}$ | | | | | |
|-----------------|---|-------------|----------------------------|-------------|--------------------------|-------------|
| | <i>Myrothecium verrucaria</i> | | <i>Alternaria oleracea</i> | | <i>Aspergillus niger</i> | |
| | O ₂ uptake | Germination | O ₂ uptake | Germination | O ₂ uptake | Germination |
| Silver | 2.9 | 0.83 | 0.64 | 0.42 | 13 | 1.3 |
| Copper | 26 | 7.6 | 17 | 5.1 | >63 | 2.0 |
| Mercury | 40 | 40 | 13 | 9 | 17 | 1.6 |
| Cadmium | >2000 | 48 | 2300 | 32 | >5000 | 1530 |
| Zinc | 406 | 130 | 3900 | 121 | >6540 | >6540 |
| Cerium | >200 | >200 | >20 | 11 | >20 | >20 |
| Sodium arsenate | >6300 | >6300 | >6300 | 1.8 | >6300 | >6300 |
| Phenol | >640 | >640 | 520 | 360 | >480 | 620 |
| Malachite green | 700 | 52 | 160 | 20 | >66 | >66 |
| Cycloheximide | >1000 | 220 | >200 | 30 | >900 | 117 |

as regards both oxygen uptake and germination, and in general copper and mercury the next most effective. This is in accord with earlier studies on spore germination (14). On the other hand, some toxicants such as cadmium, while very toxic toward spore germination, are practically without effect on oxygen uptake of *N. sitophila*, *M. verrucaria*, and *A. oleracea*.

Slope of Dosage-Response Curves

Marked concentration effects on oxygen uptake (Fig. 1 A) are shown by steep dosage-response curves (Fig. 3 A) while minor responses to concentration (Fig. 2 B) give flat dosage-response curves (Fig. 3 B). These dosage-response curves have been derived from the data of Figures 3 A and 2 A respectively. It will be seen that the wide spread of oxygen uptake curves for mercuric chloride on *M. fructicola* gives moderately steep dosage-response curves, while the close grouping of the curves of cycloheximide on *N. sitophila* gives a flat dosage-response curve. These dosage-response curves for oxygen uptake, unlike the germination curves which commonly give straight lines when plotted on logarithmic-probability paper (1, 2, 13), often break at the upper and lower ends giving sigmoid curves as shown in Figure 3 A. The slopes over the middle range of the dosage-response curves used to determine the ED₅₀ values of Tables VI and VII have been characterized for the various fungi and toxicants as steep (slope greater than 2 in log-probit units), medium (1 to 2), flat (less than 1), and no appreciable fungicidal effect at concentrations tested. An inspection of Table VIII will show that the germination curves are as steep as or steeper than the oxygen consumption curves, except with mercuric chloride on *A. niger*. This is

additional evidence that germination is a more sensitive measure of toxicity than is oxygen uptake. Also by comparison with the data of Tables VI and VII it will be seen that steepness of slope tends to be associated with the more toxic compounds.

TABLE VIII
SLOPE OF DOSAGE-RESPONSE CURVES FOR OXYGEN UPTAKE (O₂) AND GERMINATION (G) OF VARIOUS TOXICANTS ON THE SPORES OF 5 FUNGI

| Toxicant | <i>Neurospora sitophila</i> | | <i>Monilinia fruticola</i> | | <i>Myrothecium verrucaria</i> | | <i>Alternaria oleracea</i> | | <i>Aspergillus niger</i> | |
|-------------------|-----------------------------|---|----------------------------|---|-------------------------------|---|----------------------------|---|--------------------------|---|
| | O ₂ | G | O ₂ | G | O ₂ | G | O ₂ | G | O ₂ | G |
| Silver nitrate | S* | S | M | S | M | S | S | S | S | S |
| Copper sulfate | S | S | M | S | M | S | M | S | N | F |
| Mercuric chloride | S | S | S | S | S | S | S | S | S | M |
| Cadmium chloride | N | M | F | M | N | S | F | M | N | S |
| Zinc chloride | N | N | M | M | M | M | N | M | F | F |
| Cerous sulfate | N | F | F | S | N | N | N | M | N | N |
| Sodium arsenate | N | S | F | S | N | N | N | M | N | N |
| Phenol | S | S | N | N | N | N | S | S | N | S |
| Malachite green | M | M | F | S | S | S | S | S | N | N |
| Cycloheximide | N | S | F | S | N | S | N | S | N | S |

* S = Steep slope; M = Medium; F = Flat; N = No effect.

SULFUR AND OTHER WETTABLE FUNGICIDES

While it is recognized that it is desirable to use only soluble toxicants in a microrespirometer, nevertheless most fungicides of interest are practically water insoluble. Accordingly, a study has been made particularly of wettable sulfur and of some other formulated and wettable fungicides.

Wettable Sulfur

The effect of a sulfur fungicide on the oxygen uptake of the spores of seven different species of fungi was studied employing Magnetic 95 sulfur. In order to get a measurable response it was necessary to use relatively large amounts of the wettable sulfur; that is, 0.2 ml. samples of 10, 2, and 0.4 per cent suspensions or a total respectively of 20, 4, and 0.8 mg. to react with usually 10 mg. of fungus spores. These tests were run at least twice for each of seven species and consistent results were obtained. The response in oxygen uptake of the spores varied from no effect with *Stemphylium sarcinaeforme* to slight stimulation with *Aspergillus niger*, *Alternaria oleracea* and *Rhizopus nigricans*, to marked stimulation with *Cephalosporium acremonium*, *Monilinia fruticola* and *Neurospora sitophila*. Typical results with four of these species are illustrated in Figure 4. The degree of stimulation appears to be correlated with their physiological activity, i.e., initial rate of oxygen consumption. Some correlation may also be

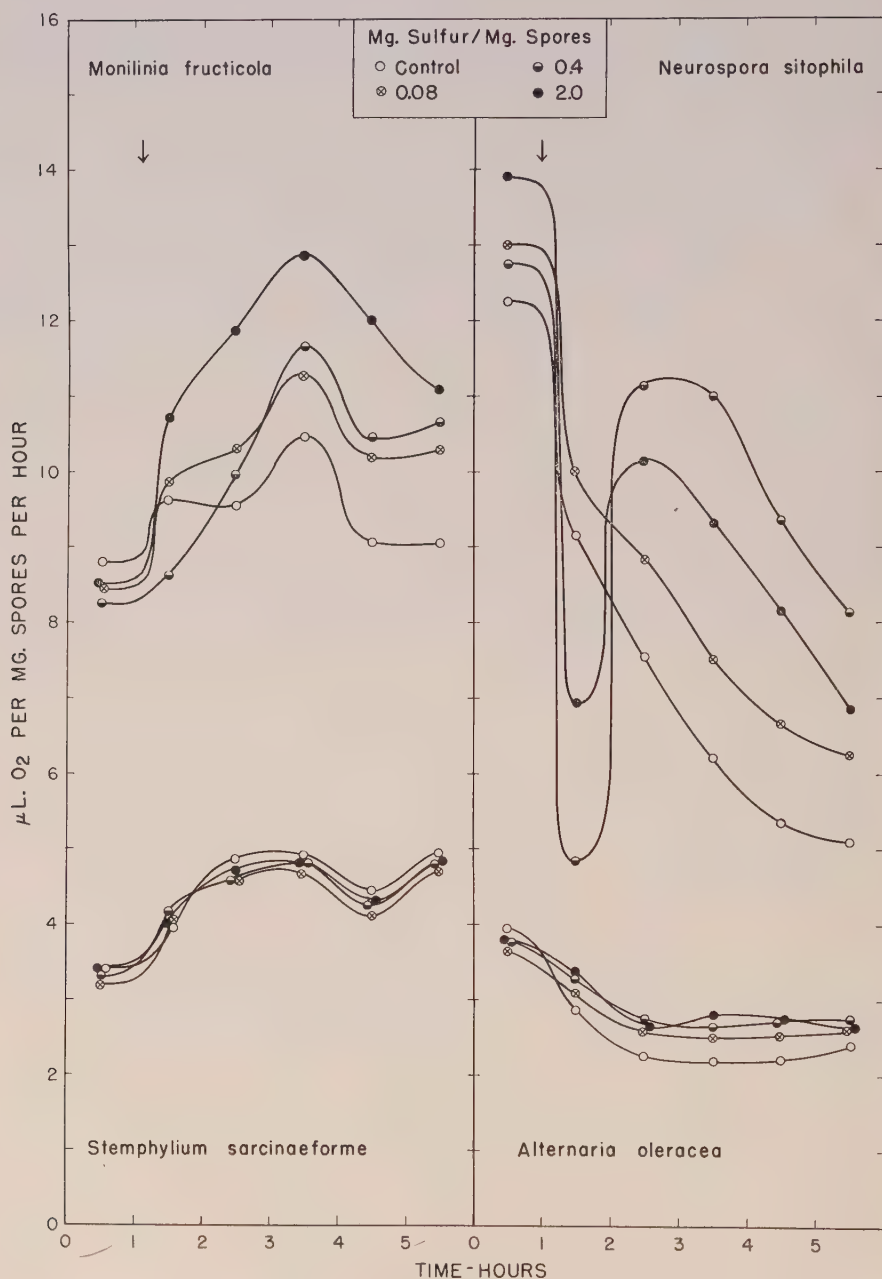


FIGURE 4. Oxygen uptake for spores of four fungi treated with a wettable sulfur (Magnetic 95). Toxicant added after first hour as indicated by arrow.

noted with their ability to reduce sulfur to hydrogen sulfide (19). The marked depression of oxygen uptake prior to stimulation of *N. sitophila* spores is to be noted in Figure 4. This was not observed with any of the other fungi. Similar effects with this fungus were obtained with some of the water soluble compounds, in particular silver nitrate as shown in Figure 2. Also the effect will be noted in later pretreatments with chloranil and dichlone though, due to the technique, the depression is at about the low point when the records begin. An examination of the spores showed no toxic effects and control germination was obtained in all cases except *Monilinia fructicola*. As is to be expected, *M. fructicola* is sensitive to sulfur and the germination percentages for the results shown in Figure 4 in descending order of amount of sulfur were 30, 55, and 86, with a control germination of 94 per cent. The stimulation from Magnetic 95 sulfur was also observed with the endogenous oxygen uptake of the more active fungus spores, though to a lesser degree.

Various preliminary tests, using in some cases radioactive S³⁵, indicated that the KOH removed most of the hydrogen sulfide produced and that adding zinc acetate with absorber papers to the second sidearm does not appreciably modify the results.

In view of the large amounts of Magnetic 95 sulfur required to produce the stimulation response, it was considered that an ingredient of the formulation might be responsible. To test this the Magnetic 95 sulfur was dissolved in carbon disulfide and the residue used in comparison with Magnetic 95 sulfur and a sample of refined flours of sulfur. The residue had a slightly depressing effect and the Magnetic 95 sulfur produced more stimulation of oxygen uptake than did the refined flours. A repetition of the test confirmed the results. Differences in particle size and wettability may influence comparisons of this kind but it is believed the effects on oxygen uptake were due to the sulfur alone.

Other Wettable Fungicides

The formulated and wettable fungicides, Spergon, Phygon, Fermate, Tersan, and Crag 341B, were studied using spores of *Neurospora sitophila*.

TABLE IX
EFFECTIVENESS OF VARIOUS FORMULATED FUNGICIDES IN REDUCING OXYGEN UPTAKE AND GERMINATION OF SPORES OF *NEUROSPORA SITOPHILA*

| Fungicide | ED50 values in μ g. active ingredient per mg. spores | |
|-----------|--|-------------|
| | Oxygen uptake | Germination |
| Spergon | 200 | 30 |
| Phygon | > 600 | < 2 |
| Fermate | > 2000 | < 20 |
| Tersan | 270 | < 10 |
| Crag 341B | 133 | < 2 |

These fungicides were tested at concentrations of 1, 0.316, 0.1, 0.0316, and 0.01 per cent of formulated material in 2 ml. in the presence of 10 mg. of spores and 1 per cent sucrose as customary. The oxygen uptake curves for Spergon, Phygon, and Tersan showed very pronounced stimulation at the lower levels, while Crag 341B and Fermate showed slight stimulation. This range of concentrations was, however, too high for spore germination and practically zero germination resulted in all cases except Spergon. Estimates of ED₅₀ values in terms of active ingredient are given in Table IX. Comparisons with similar values for the pretreatment tests given later in Table XVI show that the pretreatment oxygen uptake values in general are lower as would be expected for soluble materials. The germination results are of about the same order of magnitude.

In a similar manner a comparison was made of soluble copper sulfate and insoluble copper carbonate with the same total amounts of copper. The results are presented in Table X. It is apparent that there was some

TABLE X
EFFECT OF A SOLUBLE AND AN INSOLUBLE COPPER COMPOUND ON OXYGEN UPTAKE AND GERMINATION OF SPORES OF *NEUROSPORA SITOPHILA*

| Copper, μ g. per mg. spores | Oxygen uptake (μ l. per mg. spores in 5 hrs.) and germination (%) in presence of copper | | | |
|------------------------------------|---|-------------|-------------------------|-------------|
| | Copper sulfate | | Copper carbonate | |
| | μ l. O ₂ | Germination | μ l. O ₂ | Germination |
| Control | 23.4 | 96 | — | — |
| 0.0127 | 19.4 | 90 | 22.8 | 96 |
| 0.1274 | 31.4 | 47 | 29.4 | 90 |
| 1.274 | 12.8 | 3 | 24.8 | 56 |
| 12.74 | 6.7 | 1 | 19.4 | 17 |
| ED ₅₀ | 1.78 | 0.13 | > 12.7 | 1.91 |

stimulation of oxygen uptake at intermediate values of copper. The data and the derived ED₅₀ values indicate that the soluble copper was about 15-fold as active as the insoluble copper. This ratio, however, is very much less than that of the relative solubilities. Hence, on the basis of these results and those of the formulated fungicides above, it appears that the oxygen uptake and germination responses of difficultly soluble fungicides are greatly in excess of what might be expected from their low solubility.

PRETREATMENTS

In order to employ the pretreatment method for determining the oxygen consumption of water insoluble compounds which constitute most fungicides of practical interest, it is necessary that two factors hold. These are that oxygen uptake is proportional to the amount of spores and that

over a limited range at least the oxygen uptake and germination response is dependent on the ratio of soluble toxicant to spores. Early results had shown that a constant percentage germination resulted from a given ratio of *Monilinia fructicola* spores and copper sulfate (10). More recent results using various radioactive toxicants also indicated the dose is largely determined by the ratio of toxicant to spores (20, 21).

A direct comparison of the pretreatment method with the regular continuous exposure in the microrespirometer was made with copper sulfate and spores of *Neurospora sitophila* and *Monilinia fructicola* in amounts of 10 and 3.16 mg. of spores. Since in the regular treatment sucrose is present, a comparison of the presence and absence of sucrose in the pretreatment was included. The results are given in Table XI. It will be seen in comparing the oxygen uptake in pretreatment with sucrose against regular treatment that there is no consistent difference. Germination, however, is consistently lower in the five-hour continuous treatment. No

TABLE XI

EFFECT OF PRETREATING SPORES WITH COPPER SULFATE COMPARED TO CONTINUOUS EXPOSURE IN MICRORESPIROMETER ON OXYGEN UPTAKE (TOTAL IN 5 HRS.) AND GERMINATION (%) OF *NEUROSPORA SITOPHILA* AND *MONILINIA FRUCTICOLA*

| Fungus, mg. spores | Copper, total µg. | Pretreatment, 15 mins. | | | | Continuous exposure | |
|--------------------------------------|-------------------|------------------------|-------|--------------------|-------|---------------------|-------|
| | | Without sucrose | | With sucrose | | | |
| | | µl. O ₂ | Germ. | µl. O ₂ | Germ. | µl. O ₂ | Germ. |
| <i>Neurospora sitophila</i> , 10 mg. | Control | — | — | 319 | 89 | 311 | 90 |
| | 0.63 | 294 | 80 | 376 | 78 | 468 | 57 |
| | 2.00 | 329 | 68 | 359 | 80 | 221 | 18 |
| <i>N. sitophila</i> , 3.16 mg. | Control | — | — | 113 | 93 | 97 | 95 |
| | 0.63 | 79 | 88 | 83 | 87 | 134 | 42 |
| | 2.00 | 73 | 80 | 71 | 87 | 69 | 19 |
| <i>Monilinia fructicola</i> , 10 mg. | Control | — | — | 359 | 96 | 458 | 96 |
| | 0.63 | 319 | 90 | 259 | 76 | 264 | 67 |
| | 2.00 | 103 | 30 | 71 | 15 | 143 | 7 |
| <i>M. fructicola</i> , 3.16 mg. | Control | — | — | 107 | 97 | 140 | 89 |
| | 0.63 | 24 | 19 | 33 | 37 | 51 | 9 |
| | 2.00 | 27 | 6 | 25 | 7 | 14 | 1 |

significant difference is apparent whether sucrose is or is not present in the pretreatment. In view of this the sucrose was omitted in all succeeding pretreatments. Another comparison of pretreatment with regular treatment was made with 2-heptadecyl-2-imidazoline in 2 per cent acetone using spores of *N. sitophila*. The regular treatment did not retard oxygen uptake or germination more than the pretreatment.

In a regular Warburg determination, 3.16, 10, and 31.6 mg. of spores of

TABLE XII
EFFECT OF CONSTANT RATIOS OF COPPER SULFATE TO SPORES OF NEURO-
SPORA SITOPHILA ON OXYGEN UPTAKE AND GERMINATION

| Copper, μg. | Oxygen uptake (μl. in 5 hrs.) and germination (%) for various quantities of spores in mg. | | | | | |
|--|--|-------|--------------------|-------|--------------------|-------|
| | 3.16 | | 10 | | 31.6 | |
| | μl. O ₂ | Germ. | μl. O ₂ | Germ. | μl. O ₂ | Germ. |
| A—Copper and oxygen uptake on total basis | | | | | | |
| Control | 70 | 97 | 209 | 97 | 526 | 97 |
| 0.20 | 87 | 95 | 209 | 97 | | |
| 0.63 | | | 303 | 83 | | |
| 2.0 | | | | | 808 | 87 |
| 6.3 | 38 | 7 | | | | |
| 20 | | | 31 | 3 | | |
| 63 | | | | | 145 | 2 |
| B—Copper and oxygen uptake on basis of 10 mg. spores | | | | | | |
| Control | 221 | 97 | 209 | 97 | 167 | 97 |
| 0.63 | 275 | 95 | 303 | 83 | 255 | 87 |
| 20 | 120 | 7 | 31 | 3 | 46 | 2 |

N. sitophila were exposed to copper sulfate in amounts to give a constant ratio to the spores. The observed results on respiration and germination appear in Table XII A. In Table XII B the oxygen uptake values have been converted to a basis of 10 mg. of spores and realigned. It will be seen that both oxygen uptake and germination are more or less constant for an equivalent ratio of spores and toxicant.

In a pretreatment comparison, 10 mg. of spores of *N. sitophila* were exposed in one series to constant volume and varying concentrations of copper sulfate, and in a second series to constant concentration and varying volume to give the same total copper in the parallel series. The results shown in Table XIII indicate good agreement for both oxygen uptake and germination where the total copper is the same. This demonstrates that pretreatments may be made by varying either concentration or volume and comparable oxygen uptake rates and germination will prevail.

A study of varying volume and concentration of mercuric chloride in pretreatments of spores of *N. sitophila* to give a constant total amount of mercury is presented in Table XIV. The oxygen uptake was fairly constant but with a slight tendency to decrease with increasing volume. Germination also varied slightly and tended to increase with decreasing concentration. However, in neither case was the change great considering the 100-fold range of volume or concentration.

Since it has been shown previously with the use of radioactive tracers that a number of toxicants such as 2-heptadecyl-2-imidazoline, silver,

TABLE XIII

EFFECT OF VARYING VOLUME AND CONCENTRATION WITH CONSTANT TOTAL
AMOUNT OF COPPER SULFATE IN PRETREATMENTS OF 15 MINUTES
ON OXYGEN UPTAKE AND GERMINATION OF SPORES
OF *NEUROSPORA SITOPHILA*

| Copper | | | μ l. O ₂ per mg. spores in 5 hrs. | Germination, per cent |
|--------------|-----------------------------|--------------------|--|--------------------------|
| Vol., ml. | Concn., μ g. per ml. | Total, μ g. | | |
| 1 | 3.16 | 3.16 | 34.0 | 88 |
| 10 | 0.316 | 3.16 | 36.5 | 89 |
| 10 | 3.16 | 31.6 | 35.2 | 82 |
| 10 | 3.16 | 31.6 | 35.3 | 75 |
| 100 | 3.16 | 316 | 18.7 | 61 |
| 10 | 31.6 | 316 | 22.9 | 46 |
| Control | | | 28.8 | 94 |

cerium, and dichlone are taken up very rapidly (20, 21) and mercury fairly rapidly (21), time of exposure was not considered to be a major factor in pretreatments. However, a comparison of a one-hour pretreatment with the usual one of 15 minutes was made with spores of *Neurospora sitophila* exposed to copper sulfate, mercuric chloride and dichlone. The results are given in Table XV. It will be observed that an exposure of 60 minutes to copper or mercury reduced the oxygen uptake and germination relatively little compared to the 15-minute exposure; while with dichlone, the 60-minute treatment gave no reduction over the 15-minute treatment.

Typical examples of oxygen uptake curves for pretreatments are given in Figure 5, while ED₅₀ values for seven common fungicides on *Neuro-*

TABLE XIV

EFFECT OF VARYING CONCENTRATION AND VOLUME WITH CONSTANT TOTAL
AMOUNT OF MERCURIC CHLORIDE IN PRETREATMENTS OF 15
MINUTES ON OXYGEN UPTAKE AND GERMINATION OF
SPORES OF *NEUROSPORA SITOPHILA*

| Concn., p.p.m. | Vol., ml. | μ g. Hg per mg. spores | Oxygen uptake, μ l. per mg. spores in 5 hrs. | Germination, per cent |
|-------------------|--------------|-------------------------------|--|--------------------------|
| I | 100 | I | 6.8 | 97 |
| 10 | 10 | | 7.6 | 86 |
| 100 | I | | 8.1 | 75 |
| I | 100 | 0.316 | 7.8 | 93 |
| 10 | 10 | | 7.4 | 85 |
| 100 | I | | 8.8 | 87 |
| Control | | | 31.3 | 92 |

TABLE XV

COMPARISON OF PRETREATMENTS FOR 60 MINUTES AND FOR 15 MINUTES ON THE OXYGEN UPTAKE AND GERMINATION OF SPORES OF *NEUROSPORA SITOPHILA* EXPOSED TO COPPER SULFATE, MERCURIC CHLORIDE AND DICHLONE

| Toxicant | µg. | Pretreatment time, minutes | Oxygen uptake, µl. per mg. spores in 5 hrs. | Germination, per cent |
|----------|---------|----------------------------|---|-----------------------|
| Copper | 31.6 | 60 | 31.5 | 63 |
| | 31.6 | 15 | 35.2 | 78 |
| | Control | | 28.8 | 94 |
| Mercury | 10 | 60 | 6.7 | 78 |
| | 10 | 15 | 7.6 | 86 |
| | Control | | 31.3 | 92 |
| Dichlone | 3.16 | 60 | 35.9 | 92 |
| | 3.16 | 15 | 38.9 | 87 |
| | 10 | 60 | 31.6 | 77 |
| | 10 | 15 | 29.9 | 73 |
| | 31.6 | 60 | 23.3 | 58 |
| | 31.6 | 15 | 22.1 | 54 |
| | Control | | 29.1 | 97 |

TABLE XVI

ED₅₀ VALUES IN MICROGRAMS PER MILLIGRAM OF FRESH SPORES FOR OXYGEN UPTAKE AND GERMINATION OF FOUR SPECIES PRETREATED WITH INSOLUBLE OR UNSTABLE FUNGICIDES

| Fungicide | <i>Neurospora sitophila</i> | | <i>Monilinia fruticola</i> | |
|---------------------------------|-----------------------------|-------------|----------------------------|-------------|
| | Oxygen uptake | Germination | Oxygen uptake | Germination |
| Chloranil | > 380 | 6.4 | 3.2 | 8.6 |
| Dichlone | 316 | 1.0 | 0.37 | 0.45 |
| 2-Heptadecyl-2-imidazoline | 3.7 | 2.7 | 3.2 | 4.0 |
| Captan | > 1000 | > 1000 | 1.8 | 160 |
| Ferbam | > 320 | 26 | 36 | 105 |
| Nabam | > 100,000 | > 100,000 | > 1000 | > 1000 |
| Lime sulfur (CaS _x) | 27,000 | 22,000 | 1200 | 3900 |
| | <i>Alternaria oleracea</i> | | <i>Aspergillus niger</i> | |
| | Oxygen uptake | Germination | Oxygen uptake | Germination |
| Chloranil | 64 | 80 | > 640 | 640 |
| Dichlone | 10 | 1.0 | > 320 | 13 |
| 2-Heptadecyl-2-imidazoline | 28 | 34 | 140 | > 200 |
| Captan | 170 | 170 | > 3200 | > 1000 |
| Ferbam | > 320 | 38 | > 320 | > 320 |
| Nabam | 102,000 | 40,000 | > 100,000 | > 100,000 |
| Lime sulfur (CaS _x) | 6,900 | 960 | > 300,000 | 120,000 |

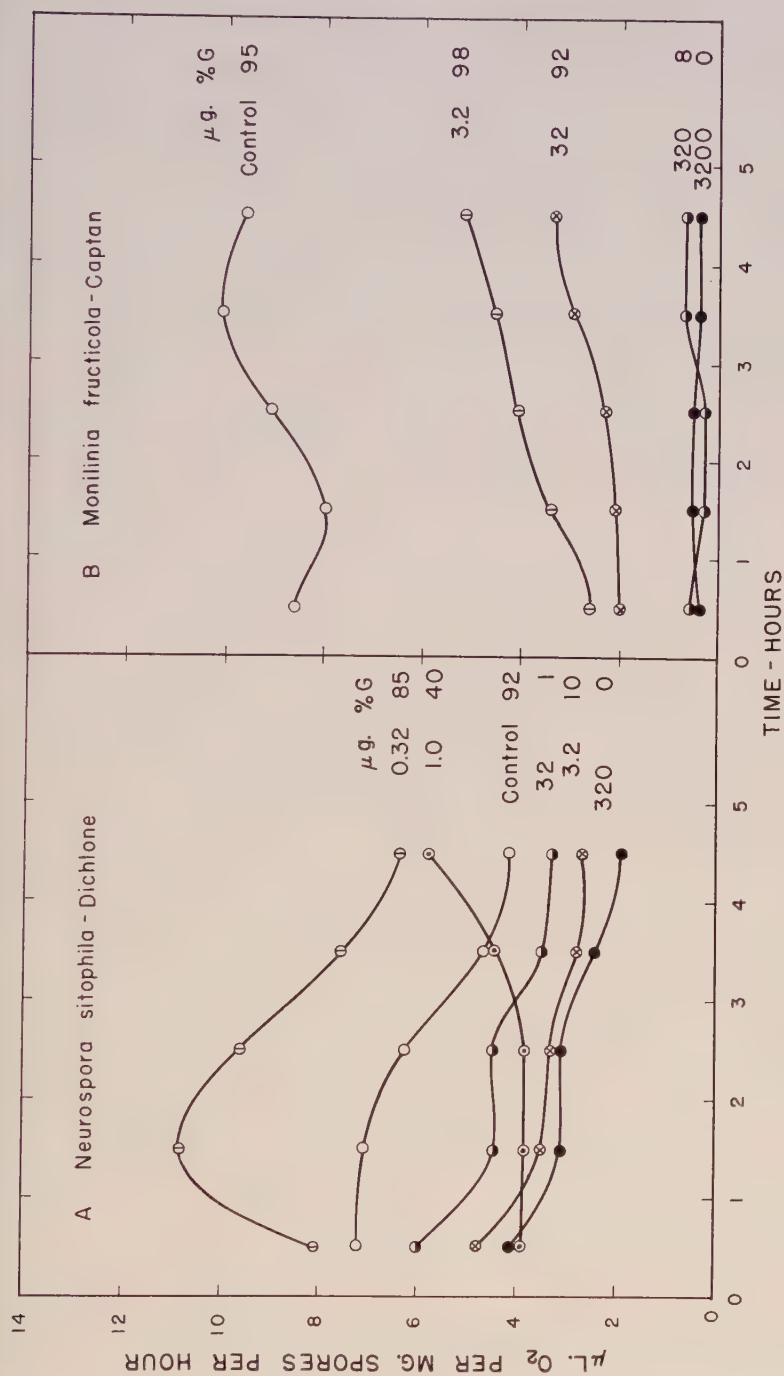


FIGURE 5. Oxygen uptake curves and germination results at 5-hour period for spores pretreated with toxicant in 2 per cent acetone solution. (A) Dichlone on *Neurospora sitophila*. (B) Captan on *Monilinia fructicola*.

spora sitophila, *Monilinia fruticola*, *Alternaria oleracea* and *Aspergillus niger* are summarized in Table XVI for oxygen uptake and germination. It will be seen that for three of the fungi, *Neurospora*, *Alternaria* and *Aspergillus*, ED₅₀ values for oxygen consumption are about equal to or greater than those for germination. This is in accord with the results with water soluble materials. However, for *Monilinia fruticola* there are a number of exceptions. The marked difference in the response of the various species to captan is to be noted. ED₅₀ values for oxygen uptake and germination for *Rhizopus nigricans*, not included in Table XVI, were > 3200 and 32 µg. per ml. of spores respectively. Nabam was without fungicidal value for any of the four organisms. This is evidence that a breakdown product is responsible for its action (9, 26).

The slope of the dosage-response curves is characterized in Table XVII. In all instances, as with the water soluble compounds, the germination curves are at least as steep as or steeper than the oxygen uptake curves. With the water soluble compounds (Table VIII) the generalization was made that high toxicity tends to be associated with steepness of slope. Two notable exceptions are evident here. The relatively nontoxic lime sulfur, at least on the basis of calcium polysulfide content, has steep dosage-response curves. Secondly, captan had a marked effect on the oxygen

TABLE XVII
SLOPE OF DOSAGE-RESPONSE CURVES FOR OXYGEN UPTAKE AND GERMINATION
OF VARIOUS FUNGICIDES ON SPORES OF FOUR FUNGI

| Fungicide | <i>Neurospora sitophila</i> | | <i>Monilinia fruticola</i> | |
|----------------------------|-----------------------------|-------------|----------------------------|-------------|
| | Oxygen uptake | Germination | Oxygen uptake | Germination |
| Chloranil | F* | S | M | M |
| Dichlone | F | S | S | S |
| 2-Heptadecyl-2-imidazoline | S | S | S | S |
| Captan | N | N | F | S |
| Ferbam | N | S | M | M |
| Nabam | N | N | N | N |
| Lime sulfur | S | S | M | S |
| | <i>Alternaria oleracea</i> | | <i>Aspergillus niger</i> | |
| | Oxygen uptake | Germination | Oxygen uptake | Germination |
| Chloranil | M | M | N | F |
| Dichlone | F | S | N | S |
| 2-Heptadecyl-2-imidazoline | M | S | M | M |
| Captan | F | F | N | F |
| Ferbam | N | S | N | N |
| Nabam | N | S | N | N |
| Lime sulfur | M | M | N | S |

* S = Steep slope; M = Medium; F = Flat; N = No effect.

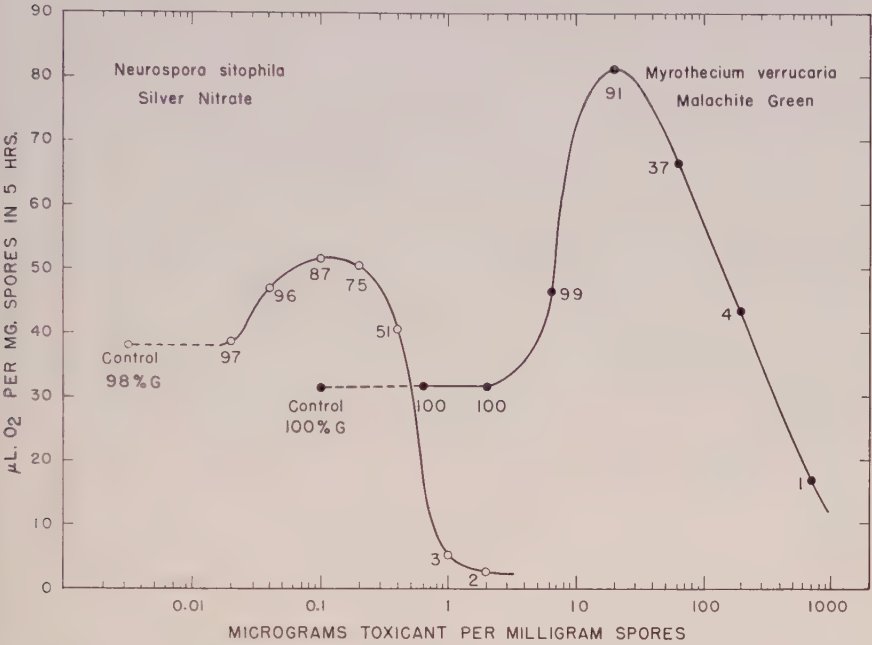


FIGURE 6. Increases in oxygen uptake induced by toxicants. Silver nitrate on *Neurospora sitophila* and malachite green on *Myrothecium verrucaria*. Numbers are per cent germination of spores at points indicated.

uptake of *M. fructicola* but the curve is notably flat, while conversely the toxicity toward germination is very much less and the curve is steep.

INCREASES IN OXYGEN UPTAKE

It was shown above that in some cases both for soluble fungicides (Fig. 2 A) and for pretreatments (Fig. 4 A) marked increases in oxygen uptake may take place. These increases show maxima at certain concentrations of toxicant as is illustrated in Figure 6 for silver nitrate on *N. sitophila* (from the original data of Fig. 2 A) and for malachite green on *Myrothecium verrucaria*. Results of this type have been summarized in Table XVIII where the maximum per cent of oxygen increase over the control is given together with the subsequent per cent germination, e.g., 36 and 89 respectively for silver nitrate on *N. sitophila*. Only those increases exceeding 10 per cent of the control are considered significant and have been recorded in Table XVIII. In three instances over 100 per cent increase was obtained, and in general the increases in oxygen uptake were not associated with marked reductions in germination.

Increases in oxygen uptake tend to be associated with particular fungi and toxicants. *Neurospora sitophila* gave increases for most toxicants and

TABLE XVIII
INCREASED OXYGEN UPTAKE INDUCED BY TOXICANTS

| Toxicant | Per cent increase of O ₂ over controls (5 hrs.) and per cent germination | | | | | | | |
|----------------------------|--|-----|-----------------------------------|-----|--------------------------------|-----|------------------------------|-----|
| | <i>Neurospora sitophila</i> | | <i>Myrothecium verrucaria</i> | | <i>Alternaria oleracea</i> | | <i>Aspergillus niger</i> | |
| Silver nitrate | 36 | 89 | Nil | Nil | Nil | Nil | 44 | 57 |
| Copper sulfate | 33 | 95 | Nil | Nil | Nil | Nil | Nil | Nil |
| Cadmium chloride | 23 | 66 | 53 | 8 | Nil | Nil | 46 | 55 |
| Cerous sulfate | 28 | 94 | 22 | 100 | Nil | Nil | 13 | 92 |
| Sodium arsenate | 26 | 72 | 68 | 100 | Nil | Nil | 24 | 90 |
| Phenol | 17 | 100 | 39 | 94 | 14 | 100 | 183 | 85 |
| Malachite green | Nil | Nil | 155 | 97 | | | 21 | 83 |
| Chloranil | 61 | 83 | — | — | Nil | Nil | Nil | Nil |
| Dichlone | 40 | 92 | — | — | Nil | Nil | 82 | 100 |
| 2-Heptadecyl-2-imidazoline | 11 | 96 | — | — | Nil | Nil | Nil | Nil |
| Captan | 82 | 91 | — | — | Nil | Nil | 48 | 71 |
| Ferbam | 16 | 31 | 64 | 100 | 19 | 59 | Nil | Nil |
| Nabam | 17 | 97 | — | — | 27 | 86 | 162 | 93 |
| Lime sulfur | 37 | 99 | — | — | Nil | Nil | 73 | 100 |

Aspergillus niger for many while *Monilinia fruticola*, not shown in Table XVIII, gave no increases. Phenol was especially effective in increasing oxygen consumption, while the toxicants, cadmium chloride, cerous sulfate sodium arsenate, ferbam and nabam, were also effective. Mercuric chloride, zinc chloride and cycloheximide, not shown, were without increased effect on any of the fungi.

DISCUSSION

There appears to be relatively little information correlating the effects of fungicides on the respiration and germination of fungus spores. Goddard (4) in his studies on the reversible heat activation of ascospores of *Neurospora tetrasperma* found that following activation of the spores by heat, and prior to germination, there was a large increase in respiration. He also observed that by treating the spores with iodoacetate or iodoacetamide all germination was prevented without greatly inhibiting the increase in respiration. It is possible that in the present studies the increase in oxygen uptake noted for *Monilinia fruticola* after about three hours may be associated with the initiation of germination. However, this increase was not apparent with *Myrothecium verrucaria*, which also germinated partially during the course of the oxygen consumption tests. Mandels and Norton (16) found that in the presence of glucose and yeast extract there was a gradual increase in the rate of oxygen uptake for the spores of *M. verrucaria* during the early stages of germination.

The depressing effect of copper sulfate and mercuric chloride on the

oxygen uptake and germination of spores of *Monilinia* (*Sclerotinia*) *fruticola* and other species was investigated by Yoder (27) while in the process of demonstrating the reversal of their toxicity by sulfhydryl-containing compounds. Comparable depressing effects on *M. fruticola* have been obtained in the present studies. Likewise, no increase in oxygen uptake in the presence of copper and mercury was noted for this fungus. However, Yoder obtained a constant high rate in oxygen uptake shortly after the addition of the sucrose which was maintained until the end of the test two to five hours later, and the present marked increase was not observed. Frampton and Marsh (3) reported that the appearance and rapid growth of the germ tube in *M. fruticola* is not accompanied by any measurable increase in oxygen consumption. Marsh (18) found that several salts would antidote the depressing effect of copper toward the conidia of *M. fruticola*, both as regards oxygen uptake and germination. The potent inhibiting effect of certain nitrophenols on the oxygen uptake of spores of *Myrothecium verrucaria* has been reported by Shirk and Byrne (23).

Mandels and Siu (17) have used measurements of oxygen consumption for the determination of the amount of growth of *Myrothecium verrucaria* and *Trichophyton mentagrophytes* on cellulose. The effectiveness of fungicides was determined by reduction in oxygen consumption which was deemed to be due to lessened growth. These authors contend that the measurement of oxygen uptake by a given amount of organism in the presence of a toxicant may not be a very good measure of the effectiveness of the toxicant. They point out as noted earlier by Goddard (5) that it has been demonstrated repeatedly that growth may be inhibited completely by exposure to physical or chemical treatments that may result in either no effect on respiration or only partial inhibition.

Insofar as some of the toxicants studied do not repress oxygen consumption but nevertheless suppress germination of the spores, it is probable that the toxic effect is exercised on some system not concerned directly with the oxygen uptake process, at least during the time interval followed in these experiments. Cadmium is an outstanding example of a toxicant which does not appreciably repress the oxygen consumption of *Neurospora sitophila*, *Myrothecium verrucaria*, or *Alternaria oleracea*, but on the other hand retards the germination. However, mercury exerts a more or less uniformly depressing effect on oxygen consumption and germination for all five species studied. With *Monilinia fruticola* only, did cadmium exert a more or less similar depressing effect. Furthermore, cadmium increased the oxygen uptake of four species, while mercury was without any such effect. Zinc, another related heavy metal, showed an appreciable depressing effect only for the germination of *Monilinia fruticola*, though the difference in response to oxygen uptake and germination, like that of cadmium, was very marked for *Alternaria oleracea*.

While studies in numerous laboratories have demonstrated the marked toxicity of nabam to fungus spores or mycelium, in all cases the effect tested was fungistatic rather than fungicidal (12). That is, nabam was allowed to remain in contact with spores or roll cultures (6) over a period of one or more days. Results with nabam are interesting in the present studies, in that exposure of fungus spores for 15 minutes in solutions of high concentration did not affect oxygen uptake nor germination. Thus, in contrast to all other fungicides tested (except possibly sulfur) no effective toxic dose is taken up in 15 minutes of exposure. It is well known that nabam is unstable and in a recent paper Ludwig, Thorn and Miller (9) have shown that products of nabam resulting from aeration, specifically ethylenethiuram monosulfide, possess a relatively high order of toxicity. Hence, it is probable that the toxicity of nabam may be due primarily to its decomposition products. In this event it could reasonably be expected that such toxic decomposition products would form from the nabam taken up by the spores. However, the results indicate that toxic products are not formed during the 15 minutes of exposure, and furthermore, that the spores do not take up sufficient quantities of the nabam to become effective subsequently. Nabam and other dithiocarbamates are believed to be effective in part through reaction with essential sulfhydryl compounds of the cell as shown in a previous communication (26) and confirmed by the recent work of Sijpesteijn and Van der Kerk (24).

In an earlier study (19) the rates of formation of hydrogen sulfide by spores exposed to sulfur were determined for a number of species. Seven of these species have also been used in these studies on oxygen uptake where the initial rates of uptake for the first hour were found to be in the following descending order: *Neurospora sitophila*, *Monilinia fructicola*, *Cephalosporium acremonium*, *Alternaria oleracea*, *Aspergillus niger*, *Stemphylium sarcinaeforme*, and *Rhizopus nigricans*. Rates of hydrogen sulfide production for the first two hours (19, Table IV) gave almost the identical order with the exception that *Cephalosporium acremonium* led in hydrogen sulfide production. The correlation of these orders of ranking is highly significant and may be an indication of the relative degree of physiological activity in general.

In the experiments with elemental sulfur, hydrogen sulfide is also produced and tests have indicated that most of the sulfide is probably absorbed in the KOH used to remove carbon dioxide. However, it is possible that some of the hydrogen sulfide given off is oxidized by the oxygen of the air to sulfur. Insofar as this may be taking place, the values for oxygen uptake in these experiments do not represent only the oxygen used in respiratory processes, and could account in part for the marked increases in oxygen uptake noted for *Neurospora sitophila* and *Monilinia fructicola*. The determination of hydrogen sulfide production in the earlier studies (19) was

made in a nitrogen atmosphere, although no effort was made to remove traces of oxygen which are present in commercially available nitrogen. For a better understanding of the nature of the reaction by which fungus spores reduce sulfur to hydrogen sulfide, it would be desirable to obtain information on the quantity of carbon dioxide produced by spores which give off hydrogen sulfide in a nitrogen atmosphere. If sulfur takes the place of oxygen then presumably one molecule of carbon dioxide should be formed for each molecule of hydrogen sulfide given off. Studies to determine this are planned.

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JOINT ACTION OF BINARY MIXTURES OF INSECTICIDES

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SUMMARY

A procedure for demonstrating similar joint action and independent joint action in mixtures of insecticides is described which is based on measurements of the time required to immobilize larvae of *Aedes aegypti* (L.). When the composition of a mixture at constant total concentration is plotted against T₅₀ (time required to immobilize 50 per cent of the larvae) the curve connecting the two pure components is convex upwards for independent joint action and concave downwards for similar joint action. This is demonstrated for a hypothetical case. Based on this method, mixtures of DDT with methoxychlor, and chlordan with heptachlor act similarly while all possible cross combinations act independently. There are some anomalies in the behavior of mixtures of these compounds with parathion but these may be caused by separate but similar reactions within the same system.

INTRODUCTION

Mixtures of insecticides may act similarly, independently, or synergistically (1). In cases of similar joint action it is assumed that the compounds act upon the same system of receptors within the insect so that one component can be substituted for another at constant ratio without changing the toxicity of the mixture. In independent joint action the components are assumed to act at different reactor sites so that the effects produced are unrelated, and the insect dies from one cause or the other rather than from the cumulative effect of the two poisons. Finney (6, p. 132) discusses a case of similar joint action involving the toxic constituents of derris root, but states (p. 137) that as far as he knows "no clear experimental demonstration of the occurrence of the simplest forms of independent action has yet been made."

It is possible that one cause of the failure to observe independent joint action is that mortality has been the criterion ordinarily employed and such a prolonged exposure period is involved that results are obscured by secondary reactions.

The development of a method for the bioassay of insecticides based on the photomigration of mosquito larvae (3) has made it possible to obtain reliable data on the effect of toxicants in a relatively short time. The larvae are treated with the test chemical and then periodically stimulated to movement with an intense light source. Ordinarily 50 per cent of the larvae will fail to respond in an hour or less, so the time during which independent joint action could be obscured by interactions within the larvae is reduced to a minimum.

With this as a basis, a study was made of the structural analogues, DDT-methoxychlor and chlordan-heptachlor, to determine whether they

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would act similarly when tested together, and independently when cross-tested. Subsequently the behaviors of mixtures of a number of other insecticides were investigated to provide background information.

MATERIALS AND METHODS

Tests were made on the following insecticides:

| | |
|--------------|---|
| DDT | 2,2-bis(<i>p</i> -chlorophenyl)-1,1,1-trichloroethane |
| Methoxychlor | 2,2-bis(<i>p</i> -methoxyphenyl)-1,1,1-trichloroethane |
| Chlordan | 1,2,4,5,6,7,8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene and related compounds |
| Heptachlor | 1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene |
| Parathion | <i>O,O</i> -diethyl <i>O-p</i> -nitrophenyl thiophosphate |
| Malathion | <i>S</i> -(1,2-dicarbethoxyethyl) <i>O,O</i> -dimethyl phosphorodithioate |
| Aldrin | 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4,5,8-dimethanonaphthalene |
| Dieldrin | 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4,5,8-dimethanonaphthalene |

Stock solutions were prepared in acetone and serially diluted to the appropriate concentrations. Aqueous suspensions were then prepared by adding 1 ml. of the final dilution to 99 ml. of water containing the test larvae. In each series of tests on binary mixtures the total concentration of insecticide was maintained constant and the proportion of one constituent to the other was varied according to the nature of the response obtained in preliminary tests. The two pure materials and three to five binary mixtures of them were included in each series. The compounds were tested at total concentrations of 1.0, 0.1 or 0.05 p.p.m., depending upon the properties of the regression curves obtained in previous experiments (5).

The time required to inhibit 50 per cent of the larvae so they no longer migrated under the influence of light (T_{50}) was determined on each suspension by the method described previously (3). The T_{50} values were then corrected back to zero time by subtracting 0.7 minute from the observed values for each hour after removal of the larvae from the nutrient solution (4).

In order to minimize fluctuations in larval resistance, each complete series of binary mixtures was tested on the same day using larvae withdrawn from the same batch. Second instar larvae of *Aedes aegypti* reared under the conditions previously described (4) were used in all experiments.

EXPERIMENTAL RESULTS

During these investigations a total of 14 series of binary mixtures was evaluated (Table I). The most extensive tests were made on the group comprising DDT and methoxychlor on one hand and heptachlor and chlordan on the other to determine whether structural analogies and differences were reflected in the type of response. In this series the total concentration of insecticide was maintained constant at 0.1 p.p.m. and the

TABLE I

TYPES OF JOINT ACTION OBTAINED ON VARIOUS BINARY MIXTURES OF INSECTICIDES

| Components of mixture | Action expected | Action obtained | Total test concentration in p.p.m. |
|-------------------------|-----------------|-----------------|------------------------------------|
| DDT-methoxychlor | Similar | Similar | 0.1 |
| DDT-heptachlor | Independent | Independent | 0.1 |
| DDT-chlordan | Independent | Independent | 0.1 |
| DDT-parathion | Independent | Similar | 0.1 |
| DDT-aldrin | Independent | Independent | 0.05 |
| Methoxychlor-heptachlor | Independent | Independent | 0.1 |
| Methoxychlor-chlordan | Independent | Independent | 0.1 |
| Methoxychlor-parathion | Independent | Independent | 0.1 |
| Parathion-malathion | Similar | Similar | 1.0 |
| Parathion-heptachlor | Independent | Similar | 0.05 |
| Parathion-aldrin | Independent | Independent | 0.05 |
| Heptachlor-chlordan | Similar | Similar | 0.1 |
| Heptachlor-aldrin | Indeterminate | Indeterminate | 0.05 |
| Aldrin-dieldrin | Similar | Similar | 0.05 |

proportion of each component varied from zero to unity in suitable steps. T_{50} values were then determined and plotted against the fraction of the most active component in the mixture.

The type of interaction in each case was arbitrarily decided by reference to a graph showing idealized cases of similar and independent joint action (Fig. 1). The following criteria were used:

Mixtures which gave curves similar to AOB where all the points were below the straight line AB , were regarded to be examples of similar joint action.

Pairs of insecticides, on which part or all of the curves were above the line AB and on which the maximum usually exceeded B , were considered to be examples of independent joint action.

Similarly, mixtures with points below A would have been considered synergistic and mixtures with points above K antagonistic. No examples in these latter categories were found.

Using these criteria, mixtures of the structural analogues DDT and methoxychlor were found to exhibit similar joint action (Fig. 2 A). This was quite pronounced since the addition of more than 50 per cent of the less effective component, methoxychlor, was required before the T_{50} of the mixture increased appreciably.

On the other hand, mixtures of DDT with heptachlor (Fig. 2 B) and DDT with chlordan were found to act independently since both gave curves with some of the points higher than the T_{50} of the least active component. Chlordan mixed with heptachlor (Fig. 3 A) showed similar joint action indicating a close relationship between structure and type of activity. The other two possible combinations, methoxychlor-heptachlor and methoxychlor-chlordan (Fig. 3 B) both showed independent action as

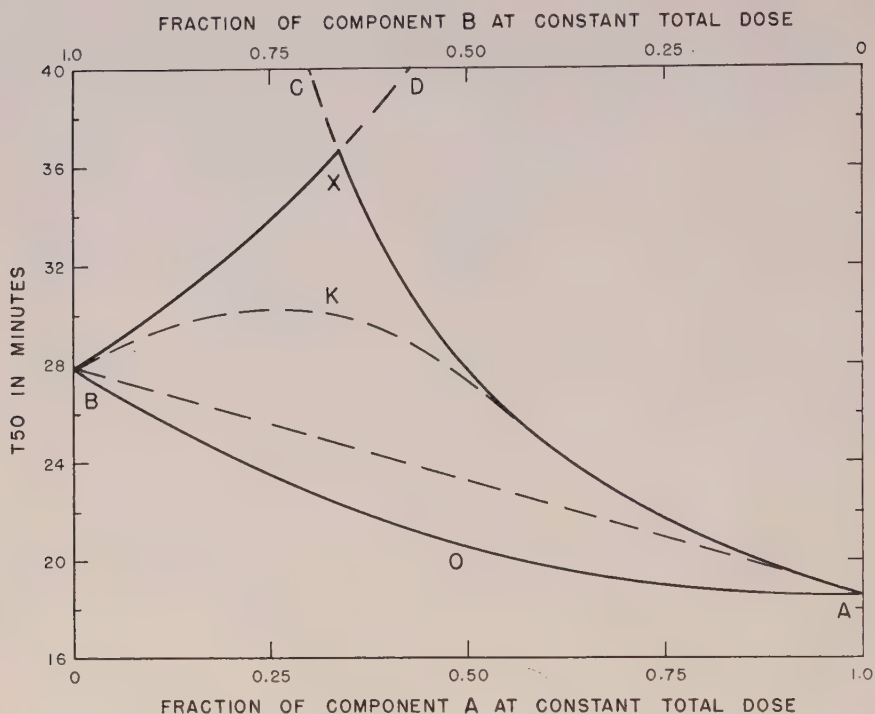


FIGURE 1. Illustration of independent and similar joint action of insecticides. *AOB*—similar joint action; *AKB*—independent joint action; *AXC*—change in T_{50} of component A with concentration; *BXD*—change in T_{50} of component B with concentration; *AB*—average value of T_{50} for a mixture.

would have been expected from structural considerations. Thus, all six binary mixtures possible in this series showed the type of joint action that would be expected in view of their structural configurations.

To determine if this correspondence would hold generally, a number of other mixtures were tested. The related materials, aldrin and dieldrin, were found to act similarly (Fig. 4 A) and aldrin and DDT independently (Fig. 4 B). The curve obtained on a mixture of aldrin and heptachlor was indeterminate but this was expected since both materials persist well on dilution and the theoretical curve corresponding to *AKB* (Fig. 1) was close to the straight line *AB*.

The results obtained on parathion in admixture with other materials were somewhat anomalous. Similar joint action was obtained with malathion as was expected but the same type of action was obtained with DDT (Fig. 5 A) and heptachlor which are structurally unrelated to parathion and act independently of each other. With methoxychlor and aldrin (Fig. 5 B), parathion exhibited independent joint action.

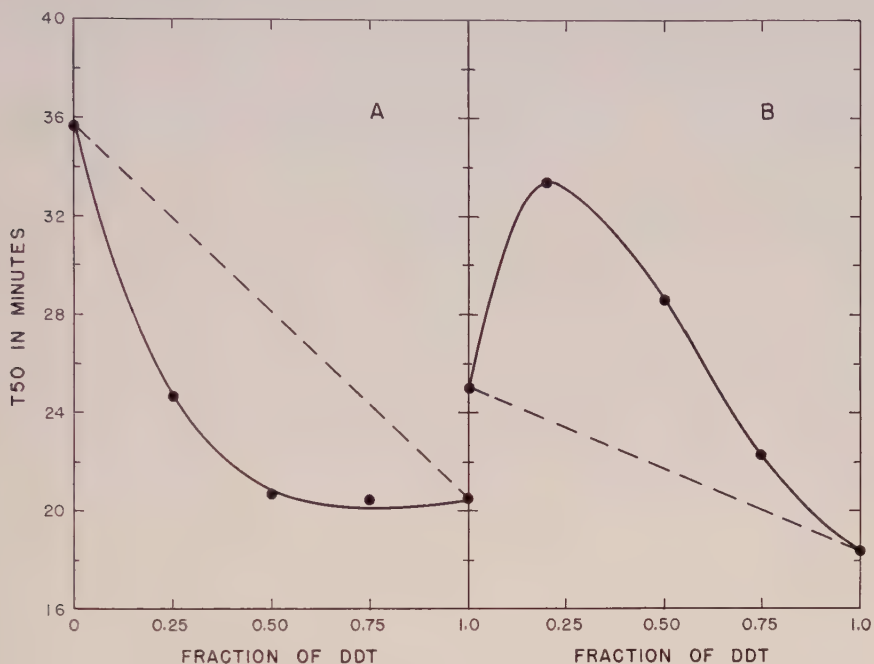


FIGURE 2. Similar and independent joint action of mixtures of insecticides. (A) DDT-methoxychlor at a total concentration of 0.1 p.p.m. (B) DDT-heptachlor at a total concentration of 0.1 p.p.m.

Of the fourteen mixtures tested, eleven of them reacted as expected. For one of them the results were indeterminate but this was caused by the nature of the regression curves relating T_{50} to concentration for these materials (5). On two of the mixtures the results were not in accord with the structural configuration of the compounds and both of these examples involved parathion.

The reproducibility of the T_{50} measurements was sufficiently precise so that classification according to joint and independent action was unequivocal in most cases. In addition, much of the data was rechecked in independent experiments with substantially the same results. In a few cases, particularly when the T_{50} values were very high, the curves were irregular but, except for the system heptachlor-aldrin, there was never any question about whether the type of action should be classified as similar or independent according to the arbitrary criteria which were established.

DISCUSSION

In an idealized case of independent action where the time vs. per cent inhibition curves for the component materials do not overlap, the T_{50} of

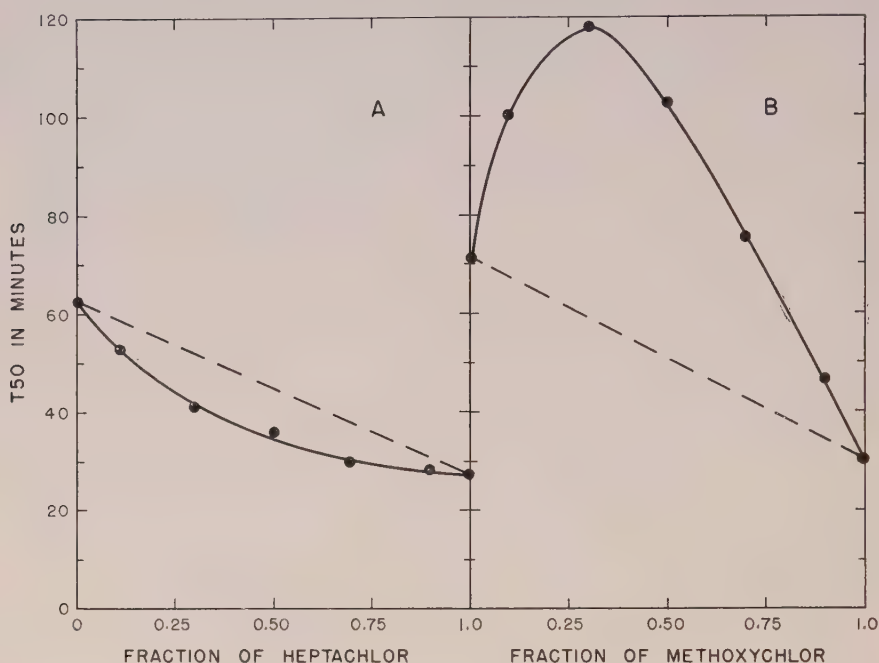


FIGURE 3. Similar and independent joint action of mixtures of insecticides. (A) Chlordan-heptachlor at a total concentration of 0.1 p.p.m. (B) Chlordan-methoxychlor at a total concentration of 0.1 p.p.m.

the mixture would be expected to be equal to the T_{50} of the most active insecticide at the concentration at which it exists in the mixture. The increase in T_{50} caused by decreasing the concentration can be calculated for each component from the regression equation

$$T_{50} = \sqrt{\tau^2 + \frac{A}{(c - c_0)^n}} \quad (1)$$

where τ is the minimum time for inactivation at infinite concentration, c_0 is the minimum concentration of insecticide that can inactivate the larvae, n and A are constants, and c is the concentration (3, 5).

When these values are calculated and plotted against percentage composition of the mixture, curves similar to AXB are obtained (Fig. 1). The point A represents the T_{50} of component A at the concentration chosen for the experiment. As the proportion of A is decreased without any supplementary contribution from component B , the T_{50} falls off along the curve AX . When X is reached the T_{50} values for both components are equal. After the point X has been passed, component A no longer contributes to the activity and the observed T_{50} is due entirely to component B .

In the example given here, component A is DDT and the curve AXC is

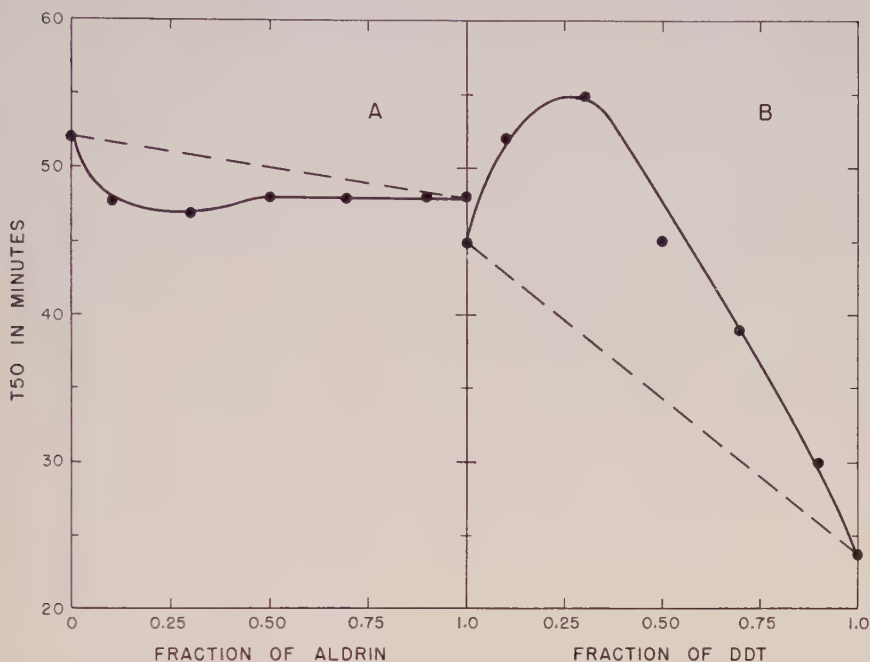


FIGURE 4. Similar and independent joint action of mixtures of insecticides. (A) Aldrin-dieldrin at a total concentration of 0.05 p.p.m. (B) Aldrin-DDT at a total concentration of 0.05 p.p.m.

the regression curve represented by equation (1) calculated from the constants given elsewhere (5). Component B is a hypothetical compound with the same regression characteristics as DDT but with one-half its activity. The curve *BXD* was obtained from equation (1) by multiplying the concentration term by a constant factor of 0.5. For illustrative purposes, this hypothetical compound was assumed to act independently of DDT.

In regions where the differences in *T*₅₀ values between the two components are large, the time vs. per cent inhibition curves will probably not overlap to any great extent since, in most cases, inactivation takes place over a relatively short interval. However, at low concentrations or where the composition of the mixture approaches *X*, overlapping will occur and the *T*₅₀ values will be lower than anticipated from the curve *AXB*.

When two materials in a mixture act independently, the proportion of individuals which will succumb is given by

$$P = P_A + P_B(1 - P_A) \quad (2)$$

where P_A is the proportion of individuals that will succumb to toxicant A, P_B is the proportion of individuals that will succumb to toxicant B, and P is the proportion that will succumb to the mixture.

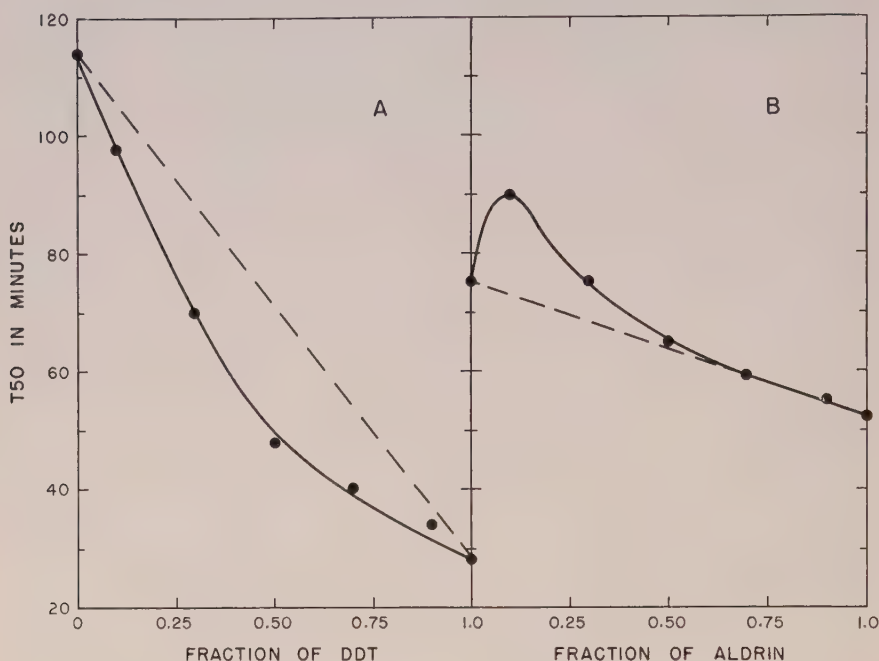


FIGURE 5. Similar and independent action with parathion mixtures showing reversal in the case of DDT. (A) Parathion-DDT at a total concentration of 0.1 p.p.m. (B) Parathion-aldrin at a total concentration of 0.05 p.p.m.

At point *X* (Fig. 1), the T_{50} values for the two components are identical when tested alone. Hence when tested in admixture 75 per cent of the larvae would be inactivated as calculated from equation (2). Thus the T_{50} value for the mixture corresponding to the point of intersection would have to be shifted downward from 36.5 minutes to some lower value. Thus *AXB* does not give a true picture of the inactivation curve even if it is assumed that there is no metabolic link at all between the sites which are being inactivated. The difference between T_{50} and T_{75} is about 6 to 7 minutes for DDT in the 35-minute range; hence, in this case, the maximum of the curve would be reduced by this amount and *AKB* would represent the adjusted curve for independent joint action.

If the two materials are assumed to act similarly, the situation is entirely different. The toxicity of mixtures is not described by the line *AB* which represents the average values for the components, but by the curve *AOB* which subtends it. This can be demonstrated for a specific case using the example above and assuming this time the occurrence of similar joint action. It is assumed here that component B can be substituted for com-

ponent A in some constant ratio ρ . In this case the regression equation (1) is modified to

$$T_{50} = \sqrt{\tau^2 + \frac{A}{\{c[p_A + (\rho)(1 - p_A)] - c_0\}^n}} \quad (3)$$

where the concentration is held constant and the decimal percentage (p_A) of A in the mixture is varied. In this example ρ is arbitrarily fixed at 0.5 since component B is assumed to have only one-half the activity of component A.

It would be very difficult to make this type of calculation in an actual case, for the regression constants of the different materials vary and it would not be possible to substitute one for the other in some constant simple ratio. However, the over-all resemblance between the experimental and theoretical curves for similar and independent joint action shows that the underlying assumptions must be approximately correct.

Even in the case of independent action it would be difficult to compare expected and actual performance on a quantitative basis. The regression constants used to compute the theoretical curves were obtained on different batches of larvae than the ones used for the joint action experiments, and normal biological variation of the test material would render close comparisons valueless. However, these calculations are useful for predicting the general type of curve that will be obtained, and the concentration at which the system should be tested. If AXC and BXD do not break sharply, the point of intersection (X) may be close to the line AB . When this is further reduced by the shift from T_{75} back to T_{50} the curve AKB may approach AB too closely to be distinguishable from it. This can sometimes be overcome by diluting the mixture to a point where the regression curves break sharply. For this reason it is easiest to demonstrate independent action on materials such as methoxychlor which lose effectiveness rapidly on dilution. The system pyrethrin-allethrin should be a particularly interesting one to study from this standpoint.

It is evident then that the shapes of the curves are dependent upon the concentrations used as well as on the interrelationships between the regression constants of the individual materials. Thus, an isolated curve will indicate only similar or independent action without giving any information on the degree of similarity or independence. The area under a curve, for example, would not be an indication of the intensiveness of this characteristic, since it will vary with concentration, intrinsic and relative activity of the components, and other factors which are not connected with the type of action. Only direct comparison of dilution data and joint action data obtained on larvae from the same batch is likely to yield information on the degree of similarity or independence.

The qualitative data so far obtained show good correspondence between chemical structure and type of activity for some compounds. Thus methoxychlor, which differs from DDT only in having two chlorine atoms replaced by methoxyl groups, acts similarly to DDT. The same is true for heptachlor and chlordan which are also closely related. When these compounds were cross-tested, independent action was obtained in each case, so evidently the two groups must be active at different receptor centers.

Structural configuration is not always a reliable guide to type of activity. Thus, parathion showed similar joint action with DDT and heptachlor, and independent joint action with methoxychlor and aldrin although it is not related, chemically, to any of these compounds.

However, DDT is known to increase the amount of free acetylcholine in nerve (8) even though it does not inhibit the enzyme cholinesterase *in vitro* (7). This may be caused by its ability to liberate the compound from its bound reserves (8). Parathion, on the other hand, is an effective inhibitor for cholinesterase so the two poisons show similar joint action in that they both increase the amount of free acetylcholine in the nerve tissue.

This does not explain why parathion has the ability to act similarly with DDT and heptachlor while these compounds act independently of one another. Parathion is generally regarded to be a nerve poison so heptachlor must have some direct effect on the nervous system despite contrary evidence on the closely related compound chlordan (2, p. 323). It may be that the difference is morphological and that DDT and heptachlor are active at two different sites in the nerve system while parathion has the ability to penetrate both of them. It is too early to say that this is definitely the case, but the problem certainly deserves further study.

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Notes

COMPARISON OF DOSAGE-RESPONSE AND TIME-RESPONSE METHODS FOR ASSESSING THE JOINT ACTION OF ANTIMETABOLITES

H. P. BURCHFIELD AND FRANK WILCOXON¹

In the preceding paper in this series (2) a method is described for assessing the joint action of binary mixtures of insecticides based on the time required for larvae of *Aedes aegypti* (L.) to lose their response to light. After this work was completed it was pointed out to one of the authors that a somewhat similar procedure had been used previously at this Institute by Frank Wilcoxon to detect synergism and antagonism in mixtures of pesticides. Although the two methods are superficially similar, they differ in many details. The mosquito larvae bioassay is based on the time required to obtain a fixed response, while the Wilcoxon test is based on the dose required to kill a certain proportion of insects or inhibit the germination of spores. Consequently the interpretation of the experimental results obtained by the two procedures differs considerably. The two procedures and the methods used for assessing the results are compared below.

Dosage-response method. The test organisms are treated in dosage series with the two pure compounds and a number of mixtures of intermediate composition. The LD₅₀ value for each composition is estimated by plotting the mortality in probit units against the logarithm of the dose and interpolating on the straight line. The reciprocals of the LD₅₀ values are then plotted against composition of the mixture and a line drawn between the points. A straight line indicates similar joint action, a curve which lies above the predicted line represents synergism, while a curve which lies below the line represents independent joint action, or in extreme cases antagonism. The two variables in this case are the composition of the mixture and the amount of material required to kill 50 per cent of the population. When LD₅₀ values are plotted directly against composition (Fig. 1) a curve below the line is obtained. Thus plotting reciprocal values aids in the interpretation of the data. This is a direct consequence of the procedures described by Bliss (1).

In the example given here component A is a hypothetical fungicide with an ED₅₀ value of 100 p.p.m., and B a fungicide with half its innate toxicity, i.e., an ED₅₀ value of 200 p.p.m., but otherwise equivalent to A in mode of action. The expected behavior of intermediate mixtures was calculated by the usual methods.

Time-response method. The test organisms are treated with the two pure

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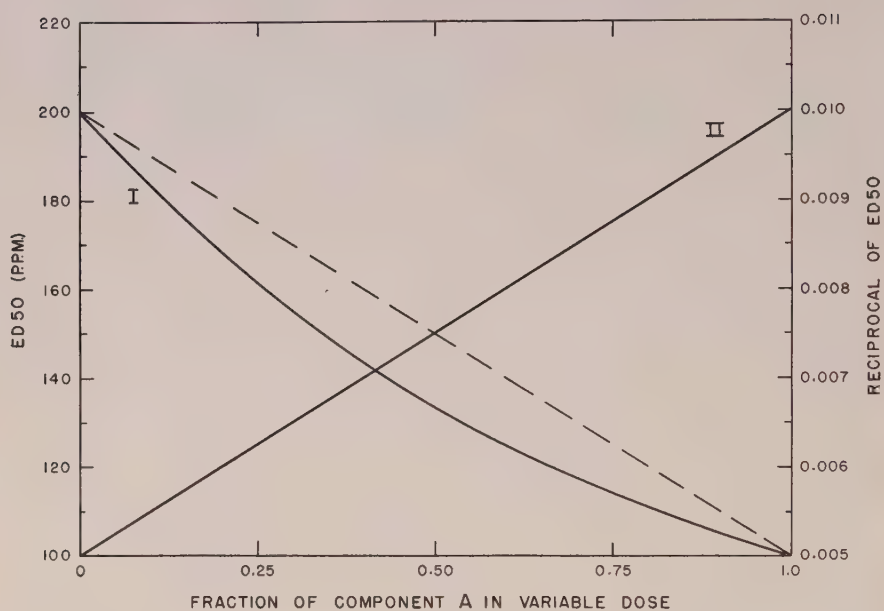


FIGURE 1. Illustration of similar joint action for a pair of fungicides where component A has twice the activity of component B. (I) Dose for fixed response vs. composition. (II) Reciprocal of dose vs. composition.

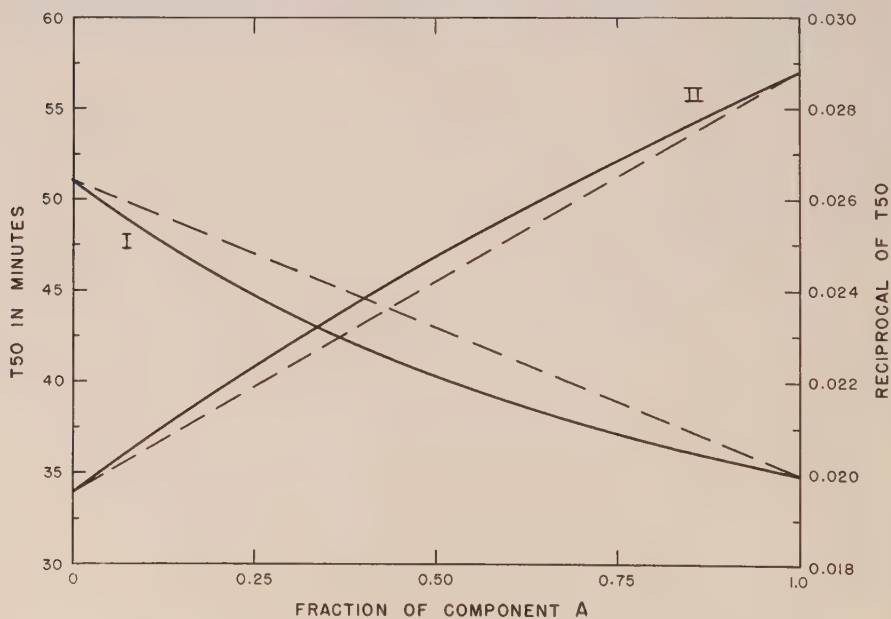


FIGURE 2. Hypothetical case of similar joint action in a mixture of two insecticides calculated from equation (1) where $\tau = 10$, $A = 100$, $c_0 = 0.01$, and $n = 1$. (I) T50 vs. composition. (II) Reciprocal of T50 vs. composition.

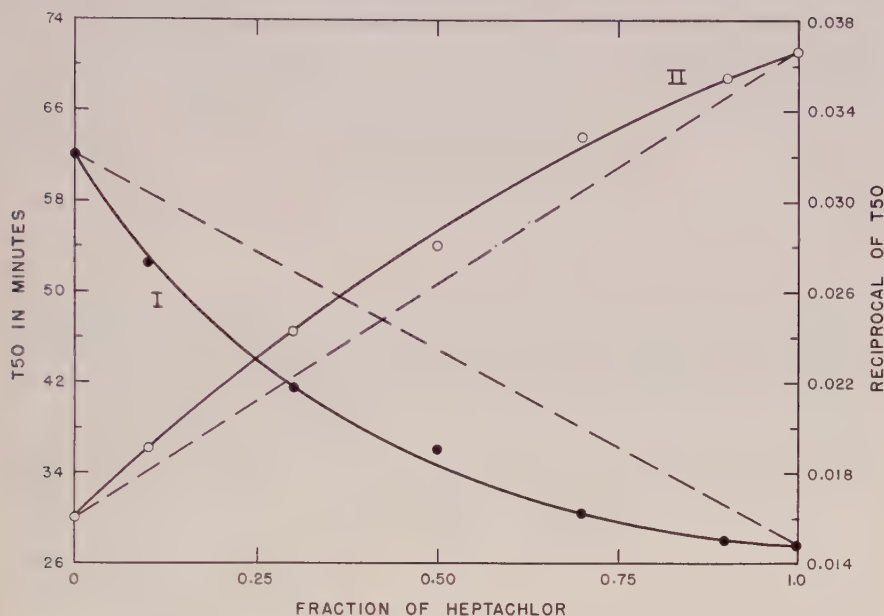


FIGURE 3. Experimental demonstration of the similar joint action of heptachlor and chlordan showing nonlinear nature of the reciprocal curve. (I) T_{50} vs. composition. (II) Reciprocal of T_{50} vs. composition.

compounds and a series of intermediate mixtures at one dose only and the time required to inactivate 50 per cent of the population determined in each case from a graph of the percentage inhibition in probit units against time. This plot is not linear on an arithmetic or a logarithmic scale nor should it be expected to be for, if X per cent of the larvae are ultimately inactivated, the curve must approach this value asymptotically.

T_{50} values are then plotted against composition in order to interpret the data (Fig. 2). A curve above the straight line connecting the T_{50} values of the two pure components is considered to represent independent joint action, and a curve below the line is considered to represent similar joint action for reasons discussed elsewhere (2). If the reciprocal of the T_{50} is plotted against composition, a straight line is not obtained either for a hypothetical case representing similar joint action (Fig. 2), or an actual case represented by a series of mixtures of chlordan with heptachlor (Fig. 3).

This failure to follow a linear relationship can be attributed to the nature of the regression equation relating time to concentration. This is

$$T_{50} = \sqrt{\tau^2 + \frac{A}{(c - c_0)^n}} \quad (1)$$

where c is the concentration, c_0 the minimum concentration that can pro-

duce 50 per cent inactivation, τ the minimum inactivation time, and n and A are constants.

If T_{50} were inversely proportional to concentration, the curve would be essentially equivalent to the one obtained by the dosage-response method since percentage composition would be plotted against a figure which corresponds directly to the amount of material required to inactivate 50 per cent of the test organisms. However, the relationship between concentration and time is more complex than this so the reciprocal curve is not linear even for similar joint action.

The essential difference between the two methods thus lies in the nature of the variables. In the dosage-response method composition of the mixture is plotted against the absolute amount of material required to obtain a standard response. In the time-response method the composition of the mixture is plotted against the time required for a fixed amount of material to give a standard response. Only in the special case where an exact inverse proportionality occurs between time and dose do the two curves become equivalent.

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A SIMPLE METHOD FOR THE DETECTION OF VOLATILE FLUORIDES IN AIR

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The method commonly used for determining gaseous fluorides at concentrations as low as one part or less of the gas per billion (10^9) parts of air (p.p.b.) requires the use of special equipment and procedures. A relatively simple method, developed in this laboratory, has proved useful for the detection and determination of approximate concentrations of volatile fluorides at levels as low as about 2 p.p.b. For this purpose a modification of Fagan's method (1) was used.

The present method utilizes dry sampling with a strip of blotting paper impregnated with a suitable reagent (2) that reacts with hydrogen fluoride (HF) and other volatile fluorides which are readily convertible into HF, such as silicon tetrafluoride (SiF_4) and fluosilicic acid (H_2SiF_6). The degree of bleaching is a function of the concentration of the gas and the duration of exposure. The procedure for impregnation of the paper is as follows: strips of white blotting paper of 0.5-mm. thickness and convenient size (5×12 cm.) are immersed for five minutes in a solution of thorium nitrate

[$\text{Th}(\text{NO}_3)_4 \cdot 4\text{H}_2\text{O}$], 1.75 grams per liter. After draining, they are immersed in a solution of sodium alizarinsulfonate, 1 gram per liter, for 10 minutes. A rose-colored lake will form on the paper surface. After a short rinsing in distilled water, the paper is dried at 40° to 60° C. During both immersions overlapping of the paper strips should be avoided to insure homogeneous impregnation.

The prepared strips, protected from rain, are hung in the desired locations for the detection of atmospheric fluorides. Half of the surface of the

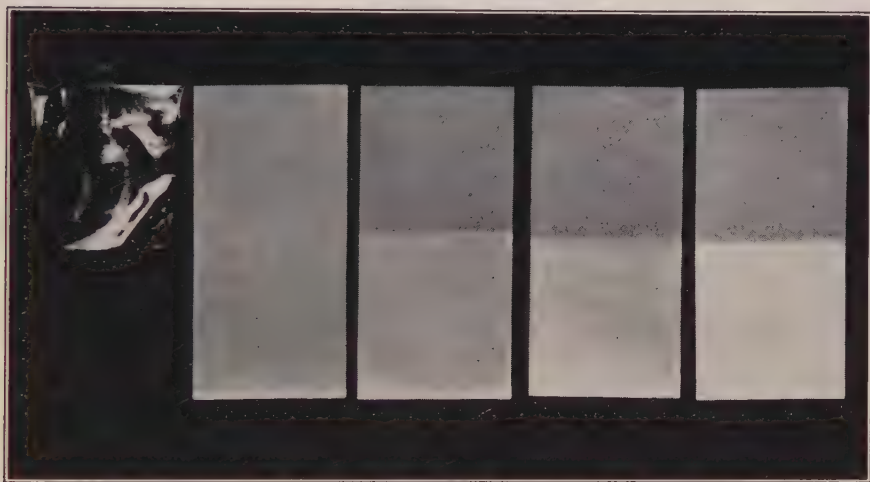


FIGURE 1. Results of exposing treated strips of blotting paper to a known concentration of HF gas. Left to right: Vinylite envelope used to cover the upper half of each strip during the test period of exposure; unexposed strip; three strips on right after exposure of the lower half for 4, 10, and 24 hours, respectively, to 60 p.p.b. HF and subsequent development of the entire strip.

strip is covered with a thin vinyl film in order to provide a control. After the absorbing medium (strips) has been exposed for a given time, it is developed by immersion for 10 to 15 minutes in a 1/100 *N* hydrochloric acid solution and the vinyl film taken off. The blotting paper is then rinsed with distilled water and dried at 40° to 60° C. Any bleaching that occurs on the exposed part, as compared with the control, constitutes a proof of the presence of volatile fluoride (as HF, H_2SiF_6 , SiF_4 , etc.) in the area. A visible bleaching of the rose-colored lake is obtained after an exposure of four hours in an atmosphere containing 60 p.p.b. by volume expressed as HF. A longer exposure will decrease the detection limit. For example, an exposure of eight days to an atmosphere containing 5 p.p.b. HF will produce a visible bleaching. The longest exposure time taken in these experiments was 16 days, after which a concentration of 2 p.p.b. could be detected. This expo-

sure time is to be considered as an approximate time limit for the detection of HF.

Fluoride concentration in unknown atmospheres may not only be detected but also approximately evaluated by standardizing the sensitive paper exposed for a given time in atmospheres of known fluoride concentration (Fig. 1). The specificity of the described sensitive paper was checked by exposing the strips to atmospheres containing sulfur dioxide, chlorine, and ammonia in concentrations of 1 to 10 p.p.m. for 24 hours without change in color. The strips were likewise unaffected by light, atmospheric moisture, and carbon dioxide during a 16-day period of exposure.

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COMPARISON OF ANTHRACNOSE FUNGI ON OAK, SYCAMORE, AND OTHER TREES^{1,2}

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SUMMARY

Seventy-five selected fungal isolates from sycamore, oak, black walnut, and American elm trees exhibiting anthracnose symptoms were compared as to nutritional requirements, host range, and morphology and were found to be of two distinct types.

Type I included isolates from sycamore, bur oak, and white oak. In comparison with Type II, they grew more slowly on culture media and produced fewer conidia. The conidia were smaller and less granular. Type II consisted of isolates from white oak, red oak, American elm, and black walnut.

The conidial stage of Type I, obtained from infected leaves, twigs and ascospores, and the associated perfect stages agreed in most details with *Gnomonia veneta* Speg. The perithecia borne on white oak leaves had longer beaks than those on sycamore. However, differences in size and shape of perithecia, asci, and ascospores were minor in nature. Isolates of Type II were not shown to be definitely associated with a perithecial stage and differ enough from *G. veneta* to mitigate against their inclusion in this species so have been referred to *Gloeosporium quercinum* West. until additional data are available on their perfect stage.

Cultures of *G. quercinum* from red oak, white oak, and walnut were infective for all three hosts as was the single isolate from American elm. One isolate from white oak infected sycamore. Cultures of *Gnomonia veneta* from sycamore infected white oak and sycamore.

G. veneta grows from sycamore leaves through the petiole and into twigs where it may incite cankers. Leaves from buds on infected twigs may become infected by limited systemic invasion. The perfect stage is considered a secondary source of inoculum. *Gloeosporium quercinum* was isolated from two- and three-year-old wood of white oak, red oak, and American elm.

Both types grow readily on potato dextrose agar but fail on a mineral salt carbohydrate medium such as Czapek's. Optimum temperature for radial spread of *Gnomonia veneta* on potato dextrose agar was 20° to 22° C. and of *Gloeosporium quercinum* 27° to 29° C.

Conidial suspensions from neither type induced anthracnose when atomized on young leaves of sycamore and white oak held in a saturated atmosphere after exposure unless leaves were wounded by removal of pubescence or oleic acid was added to spore suspensions and/or humidity was gradually reduced during incubation.

¹ From a doctoral dissertation submitted to Iowa State College, Ames, Iowa. The author gratefully acknowledges the guidance and assistance given him by his major advisor, Dr. Wendell H. Bragonier, Head of Department of Botany and Plant Pathology, and Dr. Joseph C. Gilman, Professor of Botany.

² Approved for publication as Journal Paper No. 2704 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 1047. The Iowa State Conservation Commission and the Iowa Agricultural Experiment Station, cooperating.

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Germination of both types of spores was greatly increased by suspending them in emulsions of various unsaturated fatty acids and permitting the droplets to dry gradually.

Oxygen uptake of *G. quercinum* was stimulated 250 to 300 per cent by oleic and linoleic acid. Conidia germinated best at pH 4 to 5.

INTRODUCTION

Anthracnose diseases on forest and shade trees are commonly present wherever the hosts are grown (6, 7, 8, 12, 13, 14). Both terminal and radial growth may be drastically reduced as a result of the defoliation, twig injury, and destruction of growing points caused by these diseases (2, 3, 11, 15). Seedling trees in the nursery row may be severely damaged or killed and repeated attacks on larger trees have been reported to result in death (11).

These diseases have been attributed to species of *Gnomonia* (5) whose imperfect stages are species of *Gloeosporium* and *Marssonina*. Some of the earlier investigations of sycamore and oak anthracnose raised questions as to the relationships of conidial isolates from these hosts. A study of the sexual stages indicated only one species was involved (4); however, constant differences observed in studies of conidial isolates indicated more than one fungus may be responsible for the disease (16). The disease has not been induced at will by inoculation of sycamore or white oak with fungi isolated from these hosts.

These facts prompted the critical examination of large numbers of *Gloeosporium* (conidial) isolates from sycamore, oak, walnut, and elm and their relationship to the perfect stage produced on naturally infected overwintered sycamore and white oak leaves. Further, it seemed desirable to study the morphological and physiological characteristics of single conidial and ascospore isolates in pure culture, develop suitable inoculation techniques for determining their host range, and carry out such other tests as would aid in their identification.

MATERIALS AND METHODS

SOURCE OF CULTURES

Most of the cultures used in these investigations were obtained from naturally infected leaves and twigs of sycamore and oak and to a smaller extent from elm and walnut. Pieces of infected blades, midveins, and petioles of leaves or twigs were surface disinfected by immersion in a sodium hypochlorite solution (Clorox diluted to $\frac{1}{5}$ strength) for one minute and rinsed twice in sterile distilled water. Woody tissue was placed in 95 per cent ethyl alcohol for 10 seconds, then flamed. After surface disinfection, the diseased tissue was transferred to a sterile glass slide. Small sections cut with a flamed scalpel were then planted on potato dextrose or yeast extract agar and incubated at room temperature.

Conidial isolates used in these studies were reduced to single spore cultures by the standard "biscuit cutter" method (9, p. 46). However, in making single ascospore isolates, greater care was necessary in preparation of the initial dilute spore suspension to avoid excessive bacterial contamination. Perithecia were first removed from the leaf tissue, washed several times in sterile water, and then broken open to release the spores.

Two methods were used to encourage the production of the ascogenous stage on infected oak and sycamore leaves collected in June and July. In the first method infected leaves were put between layers of moist sphagnum moss in a greenhouse flat, covered with wire screen, and incubated at about 1° C. At monthly intervals the flats were checked and enough water added to keep the leaves moist. The second method employed sterile Petri plates containing 20 ml. of 2 per cent water agar. Pieces of infected leaf tissue, surface disinfected and washed in sterile water, were planted on these plates and incubated at 5° and 10° C. The leaves were checked at monthly intervals for perithecia.

Spores from young cultures, perithecia or ascospores mounted in lactophenol were measured under oil immersion (970×) with an ocular micrometer.

From approximately 300 isolations made from leaves and twigs infected with anthracnose 75 representative cultures were selected and maintained on potato dextrose agar slants. The history of some of the representative conidial isolates used in many of the detailed studies, which were found to be of two distinct types, is as follows:

Type I (*Gnomonia veneta*), isolate G-2 was obtained from an apparently healthy midrib of a sycamore leaf. Similar isolates were obtained from infected twigs and leaves. Isolate MO-1-1 was obtained from the midrib and petiole of an infected white oak leaf collected near Holland, Michigan. Leaves on the tree were 95 per cent destroyed. A culture obtained from bur oak DO-4-1 also resembled this type.

Type II (*Gloeosporium quercinum*), isolate B-3-3 was obtained from the midrib and petiole of a white oak leaf which showed no anthracnose symptoms. The same fungus was isolated from apparently healthy new growth and one-year-old wood. The tree showed anthracnose symptoms on other leaves.

Isolate AP-103 was obtained from stem tissue of red oak that had leaf symptoms resembling oak wilt. The tree showed no leaf symptoms of anthracnose. Three other cultures of Type II were obtained from red oak trees that showed no foliage symptoms.

Culture KE-1-1 was obtained at Lacey-Keosauqua State Park in southern Iowa from a large American elm with several dead branches. Several isolations from two- and three-year-old wood yielded the same fungus which resembled the other *Gloeosporium* isolates of Type II. The symptoms on this tree were very similar to *Cephalosporium* wilt.

Gloeosporium isolate MW-1-1 was obtained from the black necrotic areas of a black walnut leaf from Pikes Peak State Park near McGregor, Iowa. A limited amount of infection occurred on the leaves in diffuse brown spots. This fungus did not fit descriptions of *Marssonina juglandis* (Lib.) Sacc. which is usually associated with leaf spot of walnut. The spores were single-celled, hyaline, and resembled the other members of Type II.

GROWTH, GERMINATION AND OXYGEN UPTAKE OF SPORES

Transfers were made to agar media to determine the nutritional requirements of representative isolates. Corn meal, nutrient dextrose, malt, potato dextrose, Czapek's, Elliott's and pea agar were prepared according to the formulae of Riker and Riker (9, pp. 26-29). Yeast infusion agar was made by using 5 grams of yeast infusion (Bacto), 20 grams of dextrose, and 20 grams of agar per liter of distilled water.

In order to determine the effect of different concentrations of dextrose on growth rate, a basal yeast infusion (Bacto) was employed. Dextrose was added at 2 to 10 per cent and the medium solidified with 2 per cent agar. Quadruplicate plates were centrally inoculated with 0.05 ml. of spore suspension from a sterile pipette. Records were taken daily on the diameter of the colonies for ten days. Similar methods were used for determining the nutritional requirements and the effect of temperature on growth on potato dextrose agar.

The effect of oleic acid, linoleic acid, dextrose, and some selected surfactants on spore germination was determined by a glass slide-germination test modified from that recommended by the American Phytopathological Society's Committee on Standardization of Fungicidal Tests (1). Six uniform droplets of spore suspension were put on each slide with a micro-pipette. The surface area of the droplets was controlled by small Vaseline rings 6 mm. in diameter stamped on the slides. After incubation for 24 hours at 25° C., 100 spores were counted in each of the six droplets. The effect of some of these chemicals on spore germination at various temperatures was determined by a similar method.

To determine the optimum hydrogen-ion concentration for germination, spores were suspended on glass slides in droplets of citric acid-phosphate buffered solutions from pH 3 to 8 and counted as described above. The effect of hydrogen-ion concentration on the growth of this isolate was determined by adjusting nutrient dextrose broth to a pH of 2.0 to 10.0 by addition of HCl and NaOH under aseptic conditions. Hydrogen-ion concentration was determined at the beginning and end of the experiment by use of a Leeds and Northrup glass electrode pH meter. Test flasks of medium were used to establish acid and alkaline charts and additions were made to the medium accordingly. Growth was determined by weight of dry mycelium.

The drying rate of droplets on glass slides was controlled in sealed chambers by lowering the relative humidity from 100 to 94 in one per cent increments by increasing the amount of NaCl per 100 g. of water from 0 to 1.5, 3.25, 5.0, 6.6 and 8.25 grams, respectively.

The respiration rate of spores was determined by measuring O₂ uptake in the Warburg constant volume respirometer. Isolate B-3-3 grown on yeast agar plates sporulated abundantly with the surface of the plate almost devoid of mycelium. The spores were removed by flooding the plate with sterile distilled water and agitating the surface with a rubber policeman. The spores were then filtered through cheesecloth to remove bits of agar or mycelial fragments and washed twice by centrifugation. The centrifuged spores were diluted with six volumes of water and 1 ml. was added to each respirometer flask. Other additions were 0.3 ml. of 0.2 molar phosphate buffer at pH 6, 0.2 ml. of 20 per cent KOH (in the center well), and 1.5 ml. of test materials to make a total volume of 3 ml. The temperature was held at 30° C. The effect of oleic acid, linoleic acid, sodium oleate and dextrose on O₂ uptake was studied.

INOCULATION

Several methods were used to determine the virulence of cultures for foliage. One-year-old white oak, red oak, black walnut, and sycamore seedlings that had been grown in the greenhouse in 8-inch pots were exposed to concentrated spore suspensions from pure cultures. These were atomized on leaf surfaces, placed on the leaves with a camel's hair brush or injected into young actively growing shoots. To encourage germination when spores were atomized on leaf surfaces, some selected surfactant or oleic acid was incorporated into the spore suspensions. To promote infection, leaves were wounded by removal of pubescence in some experiments. Plants were placed in an incubation chamber maintained at 16° to 28° C. and 100 per cent relative humidity after exposure to spores. Trees sprayed with a spore suspension were kept in the chamber until symptom expression occurred. Trees inoculated by use of a hypodermic syringe were kept in the moist chamber from 24 to 48 hours and then removed.

Free-hand sections of fresh material were cut from apparently healthy and diseased tissue and put into lactophenol to which had been added 0.5 per cent acid fuchsin and 0.5 per cent cotton blue (9, p. 68). This preparation was heated until bubbles appeared and then allowed to cool. The sections were washed with lactophenol and mounted on slides for study.

EXPERIMENTAL RESULTS

PREVALENCE OF ANTHRACNOSE DISEASES IN IOWA

During the spring of 1948, fifty counties in the State of Iowa were visited to determine the prevalence and severity of anthracnose diseases

on common shade and forest trees especially sycamore, oak, walnut, and elm.

Sycamore trees were 97 per cent infected with *Gnomonia veneta* and defoliation ranged from slight to 95 per cent. Defoliation was caused by a blight of the young leaves as they emerged from the buds. The disease first appeared on the leaves of the lower branches but progressed to higher branches later. The second crop of leaves was infected along the veins and many terminal branches were killed. Two trees, one in Waterloo and one in Marshalltown, Iowa, were without anthracnose symptoms in areas of severely defoliated trees. These observations suggest some individuals are resistant to the disease.

White oaks were similarly attacked but with less severity. In some localities 60 per cent of the leaves were infected and infection of 60 to 75 per cent of the trees was common. The disease progressed in the same manner but produced only a slight twig infection. Anthracnose was observed on other shade trees including birch, ash, hickory, and bur oak. These trees were not appreciably defoliated as a general rule.

The anthracnose diseases were not so severe in 1949. Infection appeared on sycamores throughout Iowa but very few leaves were affected during May. In the latter part of May and early June local lesions began to appear. By the middle of June some of the leaves had fallen but the trees never experienced severe leaf blight such as occurred early in May, 1948. Because of these differences, the weather records for the two years were examined.

Rainfall of 2.66 inches in April, 1948, was approximately normal and the mean temperature of 54.5° F. was 5.6° above normal while in 1949 rainfall was only 1.38 inches and the mean temperature was 49° F. In May of 1948, 2.34 inches of rainfall was below the normal of 3.96 inches but the first half of the month was marked by many days of damp, cloudy weather with high humidity and frequent showers while the temperature was slightly below normal at 59.2° F. Rainfall in May of 1949 was also below normal and in the four central counties (Story, Marshall, Polk, and Jasper) where most of the disease observations were made only 2.08 inches were recorded while temperatures were above normal and reached a peak on May 3 of over 90° F. for several days. It is believed that the warm, moist conditions during April and early May of 1948 created an environment favorable for the development of anthracnose.

COMPARISON OF ANTHRACNOSE FUNGI FROM DIFFERENT HOSTS

Morphological and cultural characteristics. In the course of making several hundred isolations during the period 1948 to 1951 from anthracnose-infected leaf, stem, and woody tissues of sycamore, oak, walnut, and elm on potato dextrose agar, it was noticed that the rate and manner of

growth differed appreciably. All of the 50 isolates obtained from sycamore, a few isolates from white oak, and a single isolate from bur oak seemed to be of one type. They grew more slowly at room temperature and were more difficult to obtain in pure culture. Cultures in Petri dishes were predominantly mycelial and produced concentric rings with spores usually developing in raised rings. Frequently, drops of yellow exudate appeared in the plates and when the cultures became older these dried to yellow waxy crusts.

Other isolates of a second type, such as the 100 from white oak, 4 from red oak and single isolates from American elm and black walnut, grew

TABLE I

SIZE OF CONIDIA FROM 10-DAY-OLD CULTURES ON POTATO DEXTROSE AGAR SEEDDED WITH ANTHRACNOSE FUNGI FROM DIFFERENT HOSTS

| Isolate measured | | Length and width of 100 conidia | |
|---|--------------|---------------------------------|-------------------|
| Code | Source | Range (μ) | Mean (μ) |
| Type I (<i>Gnomonia veneta</i>) | | | |
| Single ascospore cultures | | | |
| AO-4 | White oak | 7.9-13.0 \times 2.9-5.8 | 10.2 \times 4.7 |
| SA-1 | Sycamore | 7.2-13.0 \times 2.9-5.8 | 9.8 \times 4.8 |
| Cultures from naturally infected trees | | | |
| G-2 | Sycamore | 5.8-13.0 \times 2.9-6.5 | 9.3 \times 5.1 |
| MO-1-1 | White oak | 6.5-14.4 \times 4.3-6.5 | 9.5 \times 5.2 |
| Type II (<i>Gloeosporium quercinum</i>) | | | |
| Cultures from naturally infected trees | | | |
| B-3-3 | White oak | 11.5-18.7 \times 7.2-13.0 | 14.1 \times 9.6 |
| MA-2-3 | Red oak | 10.8-16.1 \times 4.3-8.6 | 12.6 \times 7.7 |
| MW-1-1 | Black walnut | 11.5-18.7 \times 6.5-10.8 | 15.0 \times 8.2 |
| KE-1-1 | American elm | 10.1-15.5 \times 6.0-9.2 | 12.6 \times 7.7 |

rapidly producing concentric rings of dense, black spore bodies about 4 to 8 millimeters in width separated by fine tufts of grey to white vegetative mycelium. At first these spore masses were soft and gelatinous but later, as the cultures became old and dried, crusts were formed over the spores. Typical acervuli were not produced by either type. The differences in the two types of cultures are clearly illustrated in Figure 1.

Conidia of the first type of isolates were smaller than those from Type II. Four representatives of each type were cultured on potato dextrose agar at 25° C. for 10 days and size of conidia determined. The data presented in Table I show that conidia of Type I averaged 9.7 \times 5.0 μ as compared to 13.6 \times 8.3 μ for Type II. In addition to the larger size, Type II also has a

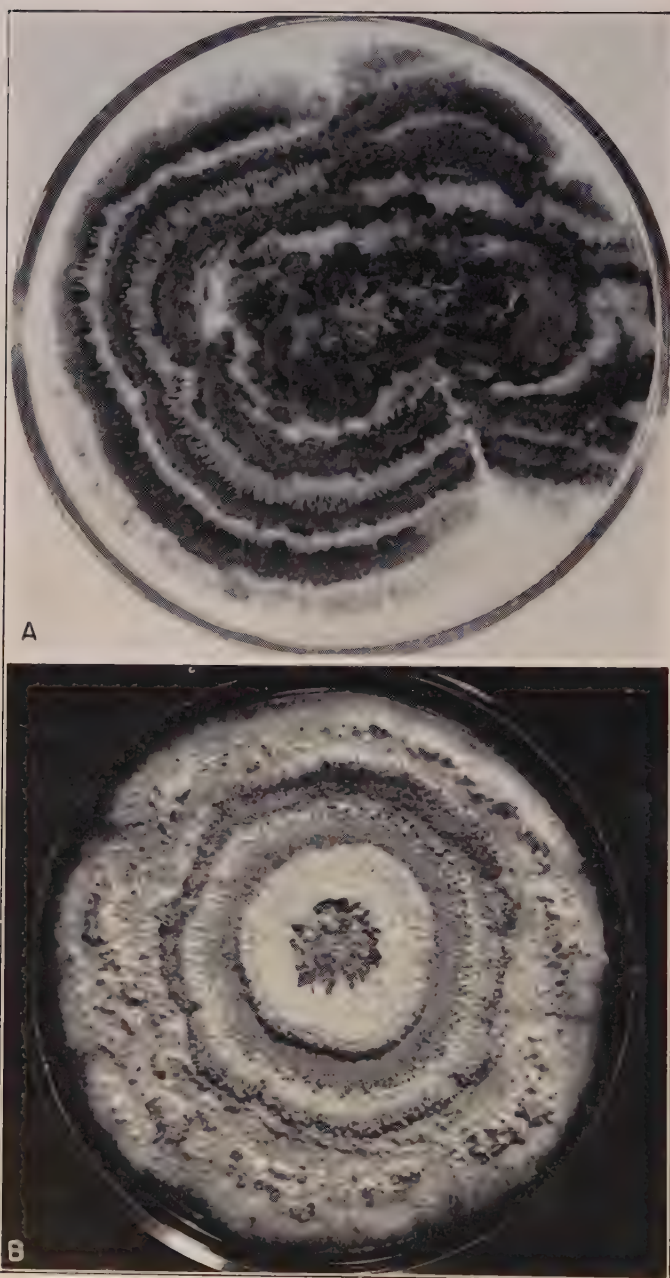


FIGURE 1. Characteristic growth habit of anthracnose fungi isolated from (A) white oak, Type II (*Gloeosporium quercinum*) and (B) sycamore, Type I (*Gnomonia veneta*) grown on potato dextrose agar.

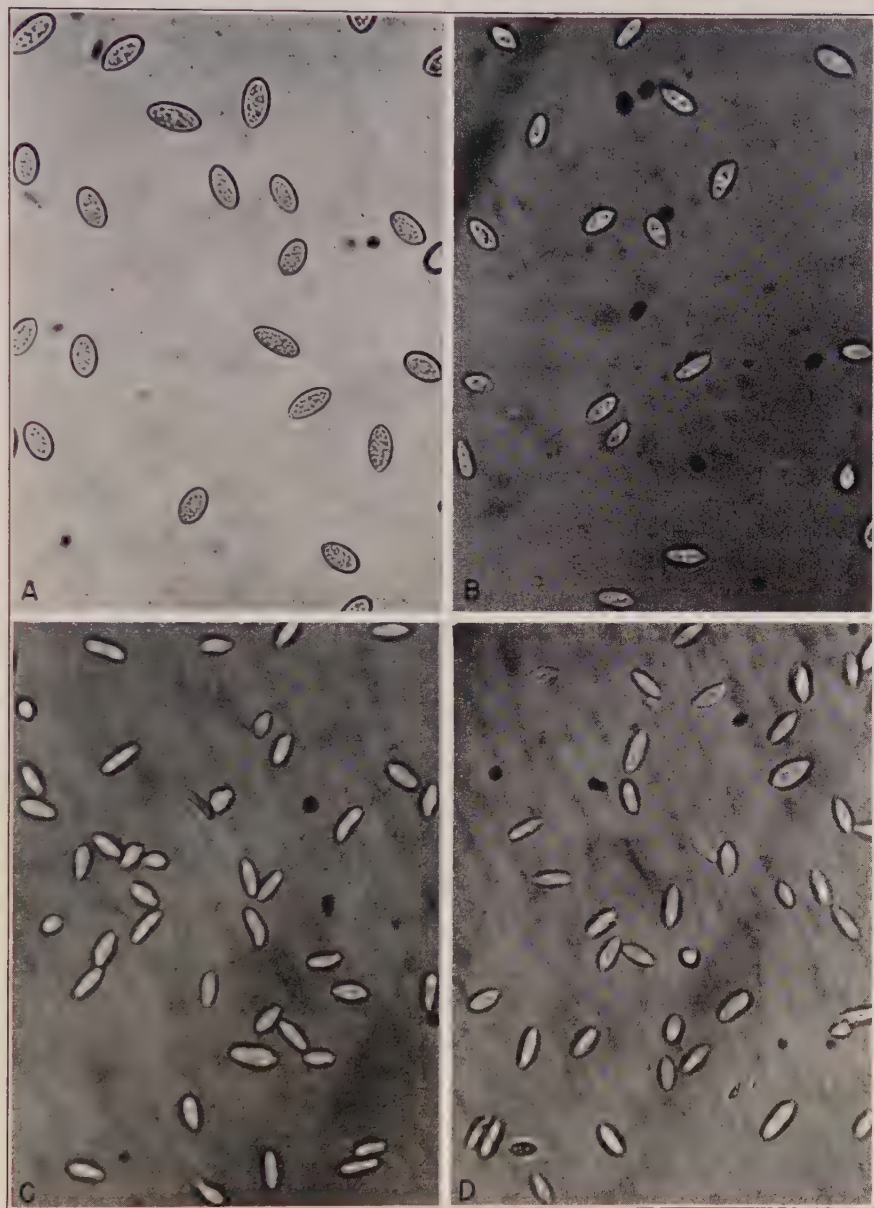


FIGURE 2. Typical conidia of *Gloeosporium quercinum* (Type II) grown on potato dextrose agar from naturally infected tissue (A). Conidia of *Gnomonia veneta* (Type I) grown on potato dextrose agar from (B) naturally infected tissue, (C) single ascospore isolates from perithecia on sycamore leaves, and (D) single ascospore isolates from perithecia on white oak leaves ($\times 450$).

more granular content as shown in Figure 2. The four isolates of each type produced conidia which were remarkably similar in appearance and size. This is particularly significant in Type I where two of the cultures had been derived from isolated ascospores and the other two from naturally infected tissue. It is concluded that the perithecial stage induced on infected leaves represented the same fungus as the mycelial stage isolated the preceding spring from sycamore and certain white oak.

Infected leaves of sycamore and white oak collected in June and July of 1949 and 1950 were kept moist with sphagnum moss at 1° C. or on water agar plates at 5° and 10° C. Perithecial initials were seen by October and mature ascospores were observed in December. Further evidence of the homogeneity of the Type I isolates was obtained because the perithecia were identical in size and differed only to the extent that the perithecial

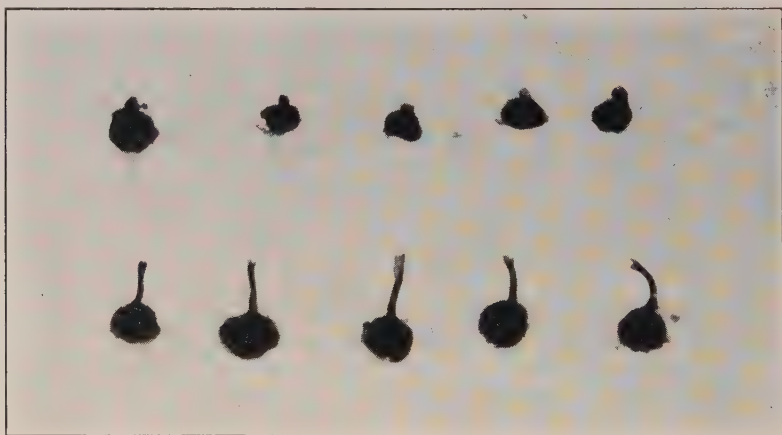


FIGURE 3. Perithecia from sycamore leaf (above) and from white oak (below).

necks, which protruded from either side of the leaves, were longer on the oak leaves (Fig. 3). The asci were very similar averaging $40.8 \times 8.2\mu$ and $41.9 \times 8.7\mu$ and containing ascospores $13.4 \times 3.7\mu$ and $14.0 \times 3.6\mu$ on the two hosts. The range of individual measurements was nearly identical. The ascospores are asymmetric in shape, have rounded ends and two cells, the smaller only one-fifth to one-quarter the length of the larger. There are eight ascospores per ascus, generally in two rows, and the thickened apex of the ascus has a pore which is surrounded by a ring (Fig. 4).

By virtue of these characteristics and all other features of the perithecia, conidia and mycelium, the Type I group of isolates qualifies as *Gnomonia veneta* Speg. Type II isolates differ sufficiently in cultural and conidial characteristics to prevent their inclusion in this species without

serious reservations. Inasmuch as their perfect stage has not been obtained to date for study, they have been classified as *Gloeosporium quercinum* West. and will be so designated henceforth in this paper.

Rate of growth on various media. Spore suspensions of a representative isolate of *Gnomonia veneta* (G-2) from sycamore and *Gloeosporium quercinum* (B-3-3) from white oak were placed aseptically in the center of Petri dishes containing test media. The average daily radial spread of four replicates of *Gnomonia veneta* during 10 days' and *Gloeosporium quer-*

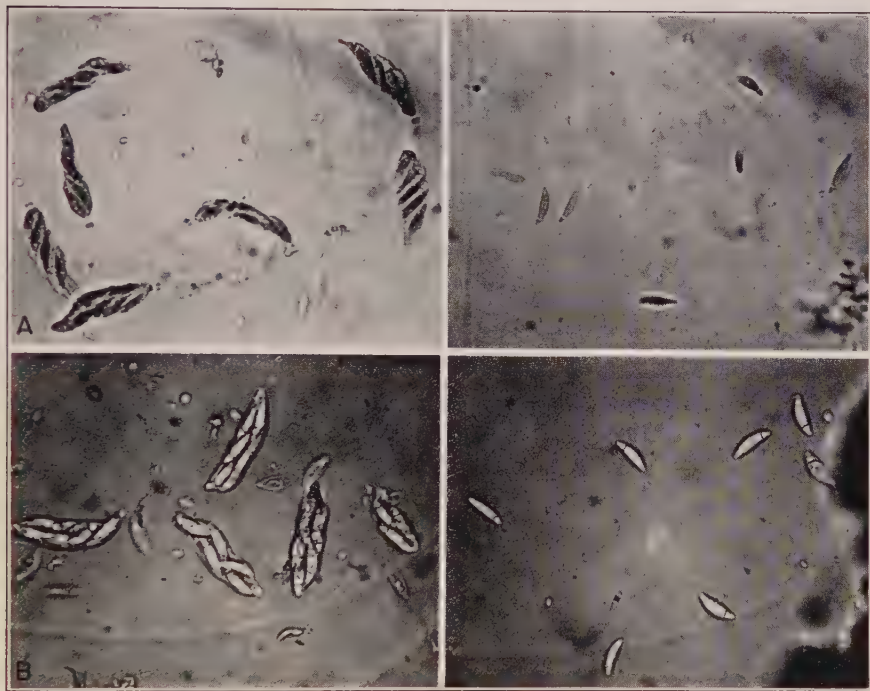


FIGURE 4. Asci (left) and ascospores (right) of *Gnomonia veneta* from perithecia produced on naturally infected, overwintered leaves of (A) sycamore and (B) white oak. ($\times 675$).

cinum during 7 days' incubation on various media is summarized in Table II. All of the natural media used were about equally suitable in supporting growth. Both isolates produced spores on all media where growth occurred. Since these isolates failed to grow on a mineral salt carbohydrate media such as Czapek's or Elliott's agar, various known growth substances were tested and results from these studies will be reported in a subsequent paper.

The effect of dextrose at 2, 4, 6, 8, and 10 per cent on the growth of one

TABLE II

GROWTH RATE OF *GLOEOSPORIUM QUERCINUM* FROM WHITE OAK AND *GNOMONIA VENETA* FROM SYCAMORE ON DIFFERENT MEDIA AT 25° C.

| Culture media used | Growth rate (mm./day) of isolates from | |
|--|---|-----------------------|
| | White oak (7 days) | Sycamore (10 days) |
| Potato dextrose agar | 8.2 | 4.0 |
| Nutrient dextrose agar | 6.4 | 4.2 |
| Yeast extract agar | 8.1 | 3.0 |
| Corn meal agar | 7.4 | 3.4 |
| Malt agar | 6.0 | 3.0 |
| Pea agar | — | 2.8 |
| Czapek's agar | 0 | <0.1 |
| Elliott's agar | 0 | — |
| 2% water agar + 0.1% asparagine | 0 | — |
| 2% water agar + 2% sucrose | 0 | — |
| 2% water agar + 2% sucrose + 0.1% asparagine | — | <0.1 |

isolate of *G. quercinum* (B-3-3) and two isolates of *Gnomonia veneta* (G-2 from sycamore and DO-4-1 from bur oak) was determined by measuring the daily radial spread in Petri plates. Centrally seeded plates in quadruplicate were held at 20° C. for 9 days and 7 days, respectively (Table III). A concentration of 8 per cent appeared to be optimum for *Gloeosporium quercinum* but the daily growth rate of *Gnomonia veneta* was still increasing at a 10 per cent concentration indicating a difference in the optimum concentration of the carbohydrate source for the growth of these two species.

Effect of temperature on growth. Increase in radial spread on potato dextrose agar, as above, determined the effect of temperature upon the growth of four isolates of *Gloeosporium quercinum* (B-3-2 white oak, MA-2-3 red oak, KE-1-1 American elm, and MW-1-1 black walnut) and one isolate of *Gnomonia veneta* (AS-81 from sycamore). Quadruplicate

TABLE III

EFFECT OF INCREASING CONCENTRATIONS OF DEXTROSE IN YEAST INFUSION AGAR ON GROWTH AT 20° C.

| Dextrose concn. (%) | Mean* daily increase (mm.) in colony size | | |
|---------------------------|--|--|--|
| | <i>Gloeosporium quercinum</i> (B-3-3 white oak) 9-day period | <i>Gnomonia veneta</i> (DO-4-1 bur oak) 7-day period | <i>Gnomonia veneta</i> (G-2 sycamore) 7-day period |
| 2 | 5.3 | 5.6 | 6.0 |
| 4 | 5.8 | 8.0 | 6.2 |
| 6 | 6.7 | 7.2 | 6.3 |
| 8 | 7.1 | 8.2 | 6.8 |
| 10 | 6.7 | 8.5 | 7.7 |
| None | 1.1 | 2.3 | 2.3 |

* Four replications.

plates were held at room temperature 48 to 72 hours before being measured and placed at 10°, 15°, 20°, 25°, 30°, and 35° C. The mean diameter of the colonies measured after 120 hours (Fig. 5) showed that all the isolates of *G. quercinum* grew best at 27° to 29° C., while the optimum temperature for sporulation was 20° C. At 20° C. a solid mass of spores was produced with mycelium developing only at the periphery. Very little sporulation occurred at 30° C. but rapid mycelial growth was still recorded. Optimum temperature for mycelial growth of *G. veneta* was 20° to 22° C. with almost

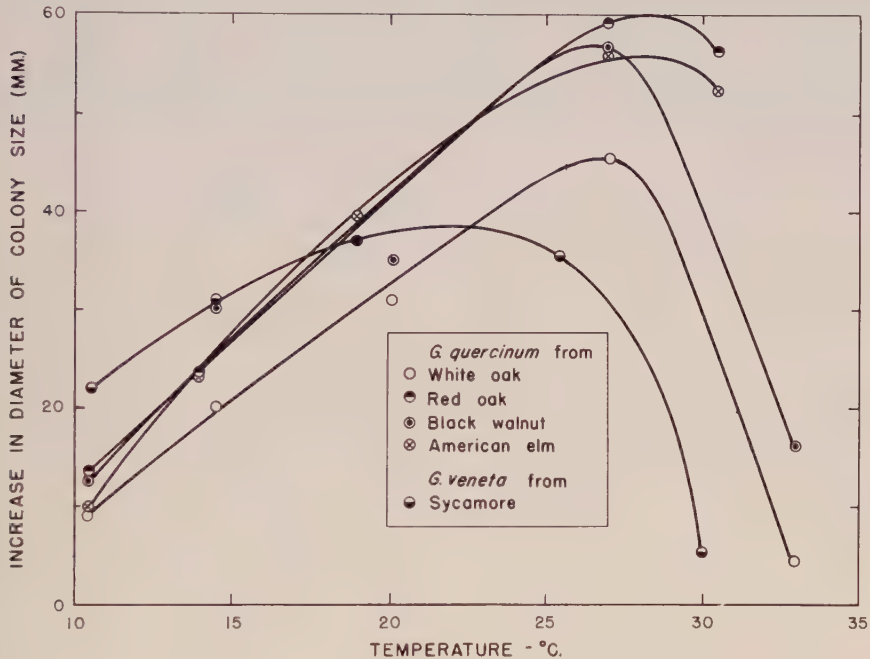


FIGURE 5. Effect of temperature on the growth of four isolates of *Gloeosporium quercinum* and one isolate of *Gnomonia veneta* within 120 hours after becoming established on potato dextrose agar.

no growth at 30° C. Maximum sporulation occurred at 15° to 20° C. but was not as profuse as the *Gloeosporium quercinum* isolates. Abundant sporulation frequently did not occur until the plates were filled with mycelial growth. These differential responses to temperature support the conclusion that the two types of isolates are physiologically different.

REQUIREMENTS FOR INFECTIVITY

Effect of fatty acids on spore germination. Infection was difficult to induce with conidial suspensions prepared from cultures obtained from

anthracnose-infected white oak and sycamore. Some success was obtained by rubbing off the pubescence, and applying a spore suspension on the leaf surfaces with a camel's hair brush before putting the seedling trees in the moist chamber. Spore suspensions atomized on leaf surfaces without wounding did not produce infection when held in a saturated atmosphere. However, in some experiments, when a wetting agent such as sodium oleate at 0.5 per cent was incorporated into the spore suspension, infection occurred. Even better results were obtained with a combination of sodium oleate and a gradual reduction of humidity in the moist chamber. In order to understand some of these effects, spore germination studies were undertaken.

No germination was obtained from spores of *G. quercinum* (B-3-3) incubated on glass slides in 0.5 per cent sodium oleate prepared from a 1

TABLE IV

EFFECT OF UNSATURATED FATTY ACIDS, SODIUM OLEATE, AND DEXTROSE ON THE GERMINATION OF GLOEOSPORIUM ISOLATE B-3-3 FROM WHITE OAK IN REPLICATE TESTS

| Treatment | Percentage germination | | | | | | Average |
|--|------------------------|----|----|----|----|----|---------|
| Check | 30 | 42 | 23 | 43 | 41 | 25 | 34.0 |
| Oleic acid 0.5% | 88 | 84 | 83 | 91 | 85 | 91 | 87.0 |
| Linoleic acid 0.5% | 81 | 63 | 59 | 79 | 81 | 64 | 71.2 |
| Dextrose 2% | 45 | 34 | 29 | 30 | 40 | 38 | 36.0 |
| Sodium oleate 0.5%, adjusted to pH 7.5 | 59 | 75 | 77 | 72 | 69 | 58 | 68.3 |
| Sodium oleate 0.5%, pH 10.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

per cent aqueous stock solution. However, when this same stock solution was tested several days later the spore germination count increased two- or threefold over the water checks. It was observed that the stock solution had changed from a clear solution to milky white. This suggested that oleic acid may be the active material responsible for the stimulatory effect. A series of tests using isolate B-3-3 revealed that a 0.5 per cent concentration of oleic acid, linoleic acid, and sodium oleate adjusted with HCl from pH 10 to 7.5, stimulated spore germination at least 35 per cent over the water checks (Table IV). When a fresh sodium oleate solution (pH 10.3) was prepared and tested no germination occurred. A 2 per cent dextrose solution usually increased spore germination over the water checks but the results were variable.

G. quercinum isolates B-3-3 (white oak) and AP-103 (red oak) were tested in a 0.5 per cent solution of sodium oleate adjusted to pH 7.5, dextrose and dextrose plus oleate. Both isolates responded the same way; the adjusted sodium oleate caused a 66 per cent increase over the water checks for isolate B-3-3 and a 41 per cent increase for isolate AP-103 from red oak (Table V).

TABLE V
GERMINATION OF SPORES OF REPRESENTATIVE CULTURES IN
DROPLETS CONTAINING DIFFERENT SUPPLEMENTS

| Culture | Code | Source | Mean percentage germination of six replicates in presence of supplement | | | |
|-------------------------------|-----------|-----------|---|------------------|------------------------|---|
| | | | None | Dextrose at 2.0% | Sodium oleate* at 0.5% | Sodium oleate* at 0.5% + dextrose at 2.0% |
| <i>Gloeosporium quercinum</i> | (B-3-3) | White oak | 19.7 | 48.7 | 86.5 | 69.0 |
| <i>Gloeosporium quercinum</i> | (AP-103) | Red oak | 5.7 | 4.1 | 47.5 | 51.8 |
| <i>Gnomonia veneta</i> | (AS-22-2) | Sycamore | 16.1 | 23.0 | 62.7 | 73.3 |
| <i>Gnomonia veneta</i> | (SO-5) | Sycamore | 3.5 | 3.0 | 30.7 | 29.7 |

* Sodium oleate adjusted to pH 7.5.

When identical tests were used on *Gnomonia veneta* isolates AS-22-2 and SO-5 from sycamore a similar response was obtained. The adjusted sodium oleate caused a 46 per cent increase over the water checks at pH 5 to 6 for isolate AS-22-2 and a 27 per cent increase for isolate SO-5 (Table V). These tests were repeated several times and increased spore germination always occurred with adjusted sodium oleate, oleic acid, and linoleic acid. Using the same method and sodium oleate as the spore germination stimulant the effect of temperature on spore germination was determined for isolate B-3-3. Maximum stimulation of germination occurred between 27° and 30° C. Optimum temperature for spore germination for this isolate agreed with the optimum temperature for mycelial growth.

TABLE VI
EFFECT OF UNSATURATED FATTY ACIDS, SODIUM OLEATE, AND DEXTROSE ON THE
OXYGEN UPTAKE OF GLOEOSPORIUM QUERCINUM ISOLATE B-3-3

| Treatment | Experiment I | | Experiment II | |
|--|-------------------|-------|-----------------|-----|
| | Q ₀₂ * | T/C** | Q ₀₂ | T/C |
| Check | 3.5 | | 2.9 | |
| Dextrose 2.0% | 8.5 | 2.4 | 5.6 | 1.9 |
| Oleic acid 0.5% | 11.4 | 3.3 | 11.6 | 4.0 |
| Linoleic acid 0.5% | 12.1 | 3.5 | 11.2 | 3.8 |
| Dextrose 2.0% + oleic acid 0.5% | 12.1 | 3.5 | 10.0 | 3.4 |
| Dextrose 2% + linoleic acid 0.5% | 14.1 | 4.0 | 12.8 | 4.4 |
| Sodium oleate 0.5%, pH 10.3 | 7.3 | 2.1 | 5.6 | 1.9 |
| Sodium oleate 0.5%, adjusted to pH 7.5 | 11.9 | 3.4 | 11.5 | 3.9 |
| Sodium oleate 0.5%, adjusted to pH 3.5 | 9.2 | 2.6 | 9.8 | 3.3 |

* Q₀₂ = μ l./hr./mg. dry wt.

** T/C = treatment/check ratio.

Effect of spore germination stimulants on oxygen uptake. The effect of oleic and linoleic acid, sodium oleate and dextrose on O_2 uptake of isolate B-3-3 was determined in the Warburg constant volume respirometer. One milliliter of spore suspension was added to each flask, and the proper amounts of the above materials were added to give final concentrations of 2 per cent for dextrose and 0.5 per cent for the unsaturated fatty acids and the sodium oleate. The results of two representative experiments show linoleic and oleic acid and sodium oleate adjusted to pH 7.5 had an identical effect by increasing the average Q_{O_2} treatment over check ratio to 3.65 (Table VI). Dextrose and sodium oleate adjusted to pH 10.3 and 3.5 had average T/C ratios of 2.1, 2.0, and 3.0 respectively. The fatty acids plus dextrose did not give an additive effect. A striking correlation exists between the increases in respiration and germination caused by these materials.

TABLE VII

EFFECT OF HYDROGEN-ION CONCENTRATION ON THE GERMINATION OF CONIDIA FROM *GLOEOSPORIUM QUERCINUM* ISOLATE B-3-3 IN REPLICATE TESTS

| pH | Percentage germination | | | | | | Average |
|----------------------------|------------------------|----|----|----|----|----|---------|
| 3 | 31 | 18 | 36 | 22 | 25 | 39 | 28.5 |
| 4 | 32 | 42 | 38 | 57 | 55 | 43 | 44.5 |
| 5 | 35 | 33 | 28 | 54 | 45 | 40 | 38.3 |
| 6 | 9 | 5 | 11 | 11 | 9 | 7 | 8.7 |
| 7 | 1 | 0 | 2 | 3 | 0 | 1 | 1.3 |
| 8 | 0 | 0 | 1 | 0 | 0 | 1 | 0.3 |
| Dist. H_2O Check, 5.3 | 39 | 56 | 38 | 41 | 40 | 41 | 42.5 |

Effect of pH on spore germination and growth. To determine optimum hydrogen-ion concentration for spore germination, *Gloeosporium quercinum* (B-3-3) was tested by using the glass slide Vaseline ring method over a pH range of 3 to 8. Optimum germination occurred at pH 4 to 5 (Table VII). This is a point of significance since sodium oleate adjusted to pH 7.5 increased germination 34 per cent over the water check at a pH of about 5.5 (Table IV). It would appear this stimulation of germination and respiration is something other than a pH effect. A distilled water check at pH 5.3 showed that the citric acid-phosphate buffer, diluted with four volumes of water, had little if any effect on germination other than that associated with change in pH. Tests were made on this isolate from white oak to determine the optimum pH range for growth in culture. Quadruplicate flasks were used at each pH level and growth was determined in milligrams of mycelial dry weight. Significant growth occurred over a range from pH 3 to 9 with the optimum between pH 5 and 7.

Effect of relative humidity and water film on spore germination. The

effect of relative humidity on spore germination was determined for isolate B-3-3. Using the glass slide Vaseline ring method, six uniform droplets of spore suspension were put on each slide and allowed to dry until free water had evaporated. One slide was then placed into each of six chambers at relative humidities of 95, 96, 97, 98, 99, and 100 per cent. After incubation for 24 hours, none of the spores germinated.

In a similar test the uniform droplets of spore suspension were not pre-dried, but placed in the chambers immediately. The drying rate of the droplets increased with decreasing relative humidities. After 24 hours at room temperature, the droplets at 100 per cent relative humidity were unchanged, while those in the 95 per cent chamber had evaporated. Germination of 91 per cent occurred in the 97 per cent chamber, 46 per cent in the 100 per cent chamber and 54 per cent in the 95 per cent chamber (Table VIII). This test was repeated three times with similar results. The thickness of water film over the spore surface and the length of time it exists are critical. This may help to explain why it has been so difficult to induce this disease using the usual greenhouse methods.

TABLE VIII

EFFECT OF RELATIVE HUMIDITY ON THE GERMINATION OF GLOEOSPORIUM QUERCINUM (B-3-3) SPORES IN DROPLETS OF WATER IN REPLICATE TESTS

| Relative humidity | Percentage germination | | | | | | Average |
|-------------------|------------------------|----|----|----|----|----|---------|
| 100 | 54 | 44 | 51 | 45 | 34 | 48 | 46.0 |
| 99 | 50 | 57 | 61 | 67 | 67 | 67 | 61.5 |
| 98 | 74 | 71 | 70 | 69 | 60 | 76 | 70.0 |
| 97 | 89 | 92 | 94 | 93 | 88 | 92 | 91.3 |
| 96 | 86 | 81 | 85 | 84 | 86 | 93 | 85.8 |
| 95 | 45 | 52 | 63 | 53 | 59 | 54 | 54.3 |

Inoculation studies. Since oleic acid and the water film stimulate spore germination, attempts were made to use this information in inoculation studies. Oleic acid was incorporated into the spore suspension as a spore germination stimulant and the relative humidity was reduced gradually in the moist chamber. The air in the incubation chamber was maintained at saturation by a fine water mist from a spray nozzle. Plants atomized with a spore suspension were put immediately into the chamber and after 4 to 6 hours the water mist was turned off. With the fan still operating to circulate the air in the chamber, the droplets on the leaf surfaces were gradually dried down. While this procedure was not entirely satisfactory it approached the conditions of the slide-germination tests in the laboratory.

To determine the effect of oleic acid (0.5 per cent final concentration) and the rate of drying on infection, one-year-old white oak seedlings with mature and young leaves were sprayed with a conidial suspension of

G. quercinum (B-3-3) from white oak. The following seven treatments were replicated three times; results were recorded ten days after inoculation:

| Treatment | Percentage of leaf surface destroyed by disease |
|---|---|
| Young leaves | |
| Spore suspension in water only | 35 |
| Spore suspension in water with oleic acid | 75 |
| Check, oleic acid in water | 0 ⁴ |
| Mature leaves | |
| Spore suspension in water only | 0 |
| Spore suspension in water with oleic acid | 60 |
| Check, oleic acid in water | 0 |
| Check, water only | 0 |

Oleic acid and a gradual drying of the spore suspension on the leaf surfaces encouraged infection and can be explained by the spore germination studies. Oleic acid was phytotoxic to young leaves but had no noticeable effect on mature leaves. Other experiments on white oak seedlings with this isolate gave similar results. Attempts to infect white oak with isolate G-2 from sycamore by this method were successful on a few trees.

By using oleic acid (0.25 per cent), the gradual drying method, removal of pubescence, and by applying spore suspensions on leaf surfaces with a camel's hair brush, inoculations with a conidial isolate from sycamore were successful in a few cases on white oak and sycamore trees.

G. quercinum isolates from red oak, white oak, and walnut induced disease in all three hosts. An isolate from elm produced disease in red oak, white oak, and walnut. Since American elm seedlings were not available, reciprocal inoculations with isolates from red oak, white oak, and walnut could not be tried on elm. *G. quercinum* from white oak produced infection in sycamore. More tests are needed under accurately controlled conditions of temperature and relative humidity to determine the combined and separate effects of these factors on infection.

STUDIES ON THE LIFE CYCLE OF ANTHRACNOSE FUNGI

The principal source of inoculum for early spring infection on sycamore is not the ascospore stage, but the conidial stage on the dead twigs. On Polk Boulevard in Des Moines, Iowa, where a spray program was conducted on sycamores (10), severe spring infection occurred, although all old leaves had been removed from under the trees. Repeated attempts to

⁴ Some injury due to oleic acid on leaf margins.

find the perfect stage on overwintered leaves in nature have been unsuccessful during the course of this study.

Viable conidia were found in the fall and early spring in the acervuli on dead sycamore twigs. Isolations from dead twigs have been successful throughout the winter, but repeated isolations from the buds of white oak and sycamore from February to April have been unsuccessful. In an attempt to follow the movement of the fungus through the host tissue, isolations were made from the following infected tissues after leaf blight symptoms began to appear: leaf blade, midvein, leaf petiole, leaf trace,



FIGURE 6. (A) Cross section of an apparently healthy sycamore petiole showing mycelium in the vessels ($\times 300$). The leaf blade was diseased. (B) Symptoms and fruiting structures of anthracnose on current season's growth of a one-year-old sycamore seedling inoculated by injecting a spore suspension of *Gnomonia veneta* into the stem (arrow).

and two inches below the node where the blighted leaf was attached. The twigs from which these isolations were made outwardly appeared healthy. However, brownish, discolored streaks which followed the leaf trace and continued down into the woody tissue between the nodes were frequently found. The fungus was recovered from each of these areas in about 60 per cent of the attempts. Free-hand sections from apparently healthy petioles of infected leaves showed the fungus in the vessels (Fig. 6 A). This indicates the fungus grows from the blade through the vessels of the petiole into the twig and may cause twig blight.

Cankers are formed on larger twigs and branches as the fungus grows in the host tissue. When longitudinal sections were made through cankers,

brown, discolored tissue was observed in the periphery of the cankered area. Isolations from the periphery of cankers on one-year-old twigs were successful, but the fungus was not recovered in clear, healthy areas below the canker. *Gnomonia veneta* was recovered from petioles of sycamore leaves which appeared to be healthy.

To determine if new growth can become infected from a canker and to prevent infection from air-borne spores, glassine bags were put over healthy twigs adjacent to a cankered twig when the buds were dormant. In May when the disease became apparent, the leaves in some of the bags, protected from infection from an outside source, showed typical anthracnose symptoms and fruiting bodies of the fungus.

In the greenhouse one-year-old sycamore seedlings were inoculated with *G. veneta* conidial and ascospore isolates by injection of spore suspensions into the one-year-old wood at the base of new growth. About two weeks after injection, disease symptoms appeared on the leaves of the new growth and several days later the fungus began to form small cankers and fruiting bodies on the dead tissue (Fig. 6 B). This method of inoculation was successful in about half of the attempts and provides added evidence that the fungus can grow from twigs to leaves. Repeated attempts to infect white oaks by this method have failed although twig infection occurs on this host.

DISCUSSION

Gnomonia veneta and *Gloeosporium quercinum* are seriously injuring the shade trees of Iowa. These fungi occur so extensively on sycamore and oak that they become of primary importance to these two species of trees. Less severe injury has been observed on walnut and elm. Evidence is presented that severity of anthracnose infection may be coupled with local climatic conditions. It was shown conclusively, for the first time, that pure cultures of these fungi will cause disease when inoculated upon leaves of healthy plants.

The data obtained in these studies help to clarify the relationships of the sexual and conidial stages of anthracnose fungi found on oak and sycamore. The sexual stages produced on overwintered leaves from white oak and sycamore were similar in all respects except the perithecial necks were long on white oak tissue and short on sycamore. The conclusion that this difference is not sufficient reason for separation of these fungi is in agreement with Edgerton's (4) findings. It is possible that beak length is dependent on the substrate and may be influenced by nutrition or other factors. Attempts to produce perithecia on sterilized sycamore and oak leaves were not successful. However, what appeared to be perithecial initials were formed, and if adequate moisture had been supplied for long-term incubation it is believed perithecia may have developed.

Conidial isolates of *Gnomonia veneta* from naturally infected tissue and conidia from single ascospores from white oak and sycamore have the same shape, size, and cultural characteristics. The conidia of all the *Gloeosporium quercinum* isolates are larger, more granular, and more uniformly shaped than those of *Gnomonia veneta*. Conidia of *Gloeosporium quercinum* were never obtained from single ascospore cultures. Both species were able to infect white oak and sycamore. Although *G. quercinum* must remain distinct until more evidence is available, there were indications throughout the course of this study that it may be only a variant of *Gnomonia veneta*. For example, isolates of *Gloeosporium quercinum* were repeatedly obtained in the spring from leaves of a number of certain white oak trees. Leaves from these same trees were overwintered and from them were obtained perithecia of *Gnomonia veneta* whose single ascospore cultures produced conidia typical of the species. Hence from one tree isolates were obtained of both types. It is possible that both species were present on this tree and simply were isolated at different times. However, it is also possible that mycelium which produces conidia typical of *Gloeosporium quercinum* may produce perithecia from which isolates typical of *Gnomonia veneta* can be obtained. If the latter possibility is true, then *Gloeosporium quercinum* is a variant of *Gnomonia veneta*.

Further evidence that a close relationship may exist between the two types of isolates was obtained on one occasion when sectoring of isolates occurred on agar containing sodium oleate. Plates inoculated with an isolate of *G. veneta* from sycamore produced conidia typical of *Gloeosporium quercinum* in the sectorized area. With the species reversed from above the same result occurred on one plate. Since these isolates had been reduced to single cells, there was no possibility of two organisms being present. Repeated attempts to duplicate these results, however, were not successful.

Westerdijk and Van Luijk (16) found larger conidia on anthracnose-infected oak leaves than on sycamore. Conidia from leaves of *Quercus pedunculata*, *Q. rubra*, and *Q. coccinea* were 12.3, 13.2, and 12.6 μ in length, respectively. These measurements agree with those found for conidia from *Quercus alba*, *Q. borealis*, *Juglans nigra*, and *Ulmus americana* which were 14.1, 12.6, 15.0, and 12.6 μ in length, respectively. It would appear the isolates they obtained from oak were similar to those designated as *Gloeosporium quercinum*, which would explain their proposal that isolates from oak and sycamore be called separate species. Their measurements on sycamore conidial isolates were in agreement with those found in this study.

Although the above fragmentary evidence is to the contrary, *Gloeosporium quercinum* must be considered distinct from *Gnomonia veneta* until it can be identified with a sexual stage. This name was proposed by Westerdijk and Van Luijk (16) for the isolates they obtained from oak.

To further establish the identity of *Gloeosporium quercinum*, leaves of white oak must be artificially infected with known isolates of the oak group and overwintered for perithecial formation.

No immediate explanation is available as to why oleic acid and linoleic acid stimulate spore germination and respiration. These materials may serve as essential growth factors in the same fashion as citrates and sucrose stimulate spore germination for many other fungi. It is more likely, however, that they change the permeability of the spore membranes. It is possible that the increase in germination obtained when droplets of spore suspension were dried slowly, may also be associated with changes in spore membranes. Spore germination stimulation cannot be explained on the basis of hydrogen-ion concentration since stimulation occurred for some materials at pH 7.5, and the optimum hydrogen-ion concentration for germination in a citric acid-phosphate buffer was pH 4 to 5.

Some of the isolates of *G. quercinum* were obtained from two- and three-year-old wood of red oak, white oak, and elm. *Gnomonia veneta* was recovered from the midrib of a seemingly healthy sycamore leaf. These fungi apparently have a limited systemic tendency in branches, twigs, and petioles and may or may not induce symptoms. For example, it was shown *Gnomonia veneta* can grow from infected sycamore leaves through petioles and cause twig blight or from infected twigs through petioles and cause leaf blight. This invasion of woody tissue is local, for although twigs are killed and cankers are formed on larger branches, the fungus is apparently restricted to these areas.

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THE SULFUR METABOLISM OF INSECTS. I. THE UTILIZATION OF SULFATE FOR THE FORMATION OF CYSTINE AND METHIONINE BY THE GERMAN COCKROACH, *BLATTELLA GERMANICA* (L.)

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SUMMARY

Adult male and immature German cockroaches, *Blattella germanica* (L.), can utilize inorganic sulfate for the production of both cystine and methionine. These two amino acids appear to be synthesized by independent routes. The degree of utilization depends on the developmental stage of the test animals; growing nymphs use sulfate at a more rapid rate than do adult males. The rate of utilization also depends on whether or not the insects are reared aseptically. The incorporation of inorganic sulfur into methionine and cystine can be carried on to a moderate degree under aseptic conditions and at a high rate under nonaseptic conditions. This intrinsic synthesis may be due to the abilities of the cockroach itself, to those of the intracellular symbionts, or to both. These faculties may be intimately related to the ability of the cockroach to survive under extremely adverse conditions.

INTRODUCTION

Although the general biology, ecology, and control of many insects are fairly well known, the basic biochemical processes related to these characteristics are imperfectly understood. Among the many groups of chemically related, biologically active substances, one of the most significant is the group of amino acids and related compounds containing sulfur. They are important not only in the production of structural proteins, but also in the synthesis and in the functioning of many of the more vital enzymes in every living organism. It is probable that they also serve in mechanisms of detoxication and resistance which have permitted insects to ameliorate and survive the effects of insecticides. These studies have been undertaken in an effort to uncover new fundamental information and ideas which will ultimately assist the toxicologist and economic entomologist in overcoming the serious resistance problem confronting them.

The German cockroach, *Blattella germanica* (L.), is one of the principal economic pests most widely used in toxicological testing of insecticides. It is easily reared and studied under closely defined laboratory conditions; hence, it was chosen to be the subject of the preliminary studies in this field.

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House (18) was the first to study specifically the cystine and methionine requirements of this insect. He concluded that, while cystine and methionine are individually dispensable for growth, cystine must be provided if individuals of this species are to proceed through normal development. The methionine nutrition of this insect was studied both in the absence and in the presence of the chemotherapeutic agent, succinyl sulfathiazole, by Noland and Baumann (24). They implied that this drug eliminated the flora of the digestive tract without affecting any synthetic powers of the cockroach itself. On the basis of results obtained under these experimental conditions, the authors concluded that methionine is required for normal growth and that any synthesis of methionine which might occur must be due to the synthetic activities of the gut flora alone.

In recent studies on cockroaches reared under aseptic conditions (15) it was found that when the insects were fed diets lacking both cystine and methionine, large amounts of both occurred free in tissue extracts and the test insects were able to survive and mature. It was pointed out that under the conditions of experiment, these substances must have been produced either by the insects themselves or by their intracellular symbionts, probably by reduction of the sulfate provided in the diet to a sulfhydryl radical which was then incorporated into cystine and methionine.

Other workers studying the metabolism of sulfur in the vertebrates have obtained varying results. Two teams of authors (9, 28) have reported that the rat is not able to synthesize cystine from sulfate; however, more recent studies have shown that the rat can perform this synthesis both from sulfide (12, 26) and, in traces, from sulfate (13). Another group of workers (20, 21) has shown that the laying hen is also capable of utilizing sulfate for the production of small amounts of cystine, but not methionine.

MATERIALS AND METHODS

Newly-hatched nymphs and 10-week-old male cockroaches of the strain maintained at the Boyce Thompson Institute were used for these experiments. The sex of the growing nymphs could not be determined at either eclosion or the time they were sacrificed for assay. However, five-week-old nymphs would not have progressed in their sexual differentiation to any degree and may therefore be considered to be representative of the immature period in development. The adult male gains little or no weight after attaining maturity; therefore, the young adult male may be considered for these purposes to be in a state of relative nutritional and metabolic balance.

The insects were fed a sterile diet lacking both cystine and methionine; this synthetic diet was basically the same as the synthetic control diet described by House (17). The only difference lay in the fact that both the

sulfur amino acids were withheld; the diet was the identical lot of diet used in earlier studies on sulfur nutrition (15) and was sterile at the time it was offered to insects in the present experiments.

The sulfate ion, the hypothetical source of sulfur in the synthesis of cystine and methionine, was provided in the form of trace amounts of $\text{H}_2\text{S}^{35}\text{O}_4$. Na_2SO_4 provided in the nutritional salt mixture incorporated into the diet served as a carrier. The labeled H_2SO_4 was offered *ad libitum* to the test animals as a solution in deionized water; the concentration of this solution was 0.0136 millicurie per milliliter. Water supplies were replenished in the cages as necessary from the original stock solution prepared at the beginning of experimental period. Thus physical decay was kept uniform throughout all feeding solutions.

The cockroaches were confined in one-liter beakers in five groups of approximately 50 each. Filter paper cones used to provide extra surface area for the insects were changed weekly to prevent excessive accumulation of excretory products. Periodically, samples of 10 cockroaches each were withdrawn, two from each of five jars, for use in chemical and radiological analyses. At the end of 21 days the solutions of $\text{H}_2\text{S}^{35}\text{O}_4$ were replaced by deionized water. Sampling and analysis were continued in order that data could be obtained on the approximate biological half-life of S^{35} in this insect.

In a parallel series of experiments two groups of nymphs were hatched, one from untreated egg capsules, the other from egg capsules sterilized as previously described (15). The former were reared nonaseptically and the latter, aseptically, under the same general conditions described above, until they were five weeks of age. They were then sacrificed for chromatographic and radiological assay.

HYDROLYSIS AND PREPARATION FOR RADIOASSAY

Each of the samples of 10 cockroaches withdrawn from the colonies was subjected to a standard method of preparation and analysis. This protocol was based largely on methods in common use in studies of sulfur metabolism (6, 7).

The digestive tract of each of the 10 insects in a given sample was removed. The last segment of the abdomen was snipped off; the head was grasped with forceps, and, with a gentle pulling motion, the head and the attached digestive tract was separated from the body. The 10 carcasses comprising the sample were then ground in acetone in a glass mortar. The resulting brei was dried in acetone for 24 hours. Subsequently, lipids present in the dry residue were extracted in anhydrous ethyl ether over a period of at least 24 hours. The fat-free residue was dried, weighed, and hydrolyzed in 6 *N* HCl for 15 to 16 hours. The hydrolysate was then evaporated and taken up in 5.0 ml. of hot water slightly acidified with HCl. The

activity of the hydrolysate was measured using the equipment described below. The results were used to calculate the activity of total sulfur.

Unlabeled Na_2SO_4 was added to the remainder of the hydrolysate to serve as a carrier in further manipulations. The hydrolysate was brought to the boiling point, and the sulfate was precipitated by adding barium chloride to a slight excess. The resulting suspensions were allowed to stand at least 15 to 16 hours and were then centrifuged. The supernatant solutions were transferred to fresh tubes, the excess barium was precipitated with dilute sulfuric acid, and the new suspension was centrifuged. The final supernatant liquid was evaporated to dryness. The residue was then taken up in 3.0 ml. of 10 per cent (vol./vol.) isopropyl alcohol. Triplicate aliquots of 6 microliters from each sample were placed in planchets for radiological assay. The radioactivity of these aliquots was determined with conventional scaling equipment and end-window type Geiger counting tubes with thin windows.

The results obtained from these assays were used to calculate the radioactivity of the organic sulfur fractions of the samples.

The values showing the activities of the total sulfur and organic sulfur fractions were corrected for the removed aliquots and for losses due to radioactive decay. No corrections for self absorption or coincidence were necessary since the size of the aliquots was regulated in such a way as to eliminate these factors. Finally, the corrected data were reduced to terms of activity per milligram of water-free, fat-free body weight of cockroach. These data were then used to plot a curve describing the relationship which existed between the time of exposure of the insects and the level of activity attained in their body tissues.

STUDY OF ANALYTICAL LOSS

In view of the known tendencies of cystine and methionine to be destroyed to some extent during the standard methods of hydrolysis (5) it was necessary to obtain, if possible, an estimate of the approximate losses which had been encountered in the use of these methods.

A large sample of organic sulfur in the form of cystine and methionine labeled with S^{35} was prepared free of inorganic sulfur by means of the methods described above. This sample containing a known S^{35} activity per unit volume served as a stock solution for experiments designed to estimate the losses of organic sulfur to be expected in the analytical methods.

First a series of tests was carried out to determine the efficiency of the methods used to remove the $\text{SO}_4^{=}$ from the test samples. Samples containing labeled $\text{SO}_4^{=}$ were hydrolyzed in hydrochloric acid and precipitated with barium chloride in the usual manner. Radiological assays before and after hydrolysis were used to estimate the efficiency of the methods of $\text{SO}_4^{=}$ precipitation. A check was also made of the purity of the stock solution.

Samples of this solution were treated with barium chloride to show whether or not there was any appreciable contamination of the organic stock solution by barium-precipitable ions. Small samples were removed from this stock solution and carried again through the analytical procedure. The activity of these samples was assayed before and after each major manipulation; the losses incurred at each step were then calculated.

CHROMATOGRAPHY

Two hundred milligrams of dry, ether extracted cockroach body solids (nitrogen = 15.1 per cent) were hydrolyzed with 20 ml. 6 *N* HCl for 24 hours. The hydrolysate was evaporated to dryness three times to remove the excess HCl; the residue was taken up in 5 ml. of water. The hydrolysate was then applied to Whatman No. 3 filter paper in 10 microliter aliquots as a series of spots arranged 2 cm. apart in a line 2.5 cm. from the edge of the paper. The amino acids were separated using the *n*-butanol-acetic acid-water mixture described by Block (4) and Block, Stekol, and Loosli (7). Sample strips were removed and treated with ninhydrin to locate the cystine and methionine bands. The developed paper sheets were each cut into five strips as indicated in Table II. The amino acids were concentrated at the center of each strip by chromatographing with water and then eluted from the paper with hot water. The eluate was then evaporated to dryness, diluted to standard volume, dispensed into planchets, and counted.

S³⁵-labeled cystine of the hydrolysate was precipitated in the presence of 100 mg. of cold carrier cystine from the dilute hydrochloric acid solution by the addition of sodium acetate. S³⁵-labeled methionine was recrystallized in the presence of 100 mg. of cold carrier methionine from an equal volume of the hydrolysate by adding ethanol to produce a final concentration of 85 per cent. Samples of these two products were chromatographed to check their purity, and 1-mg. samples were radioassayed by standard procedures.

RESULTS AND DISCUSSION

The results of the periodic assay of the experimental cockroaches for their content of radioactive sulfur are summarized in Figure 1. The organic sulfur fraction is defined as the sulfur-containing compounds remaining in solution after acid hydrolysis and precipitation of inorganic sulfates as BaSO₄. The primary effects of the hydrolysis are the breaking up of the proteins to yield amino acids and the destruction of the ethereal sulfates to yield inorganic sulfates and sulfur-free organic moieties. Since a high level of radioactivity occurs in the S³⁵O₄⁼-free solution, it is clear that a considerable amount of the sulfur has been converted from the inorganic to the organic form. These procedures do not lead to any information concerning the forms in which this organic sulfur exists. The upper curve in Figure 1 shows the rate of incorporation of all forms of S³⁵ into the insect

organism. The lower curve represents the rate of formation of organic S^{35} -bearing compounds in the insect body. This curve is based on observations uncorrected for analytical losses and represents the observed organic sulfur activity.

Both of the curves are characterized by a rapid rise in the first five days of the experiment; this suggests that the rate of assimilation of $SO_4^{=}$ is quite rapid. The initial rise is followed by a tapering of the curves toward plateaus indicating that the anabolic reactions involved tend to reach some form of balance with the related catabolic processes. The results indicate that this species of cockroach is capable of converting at least 72

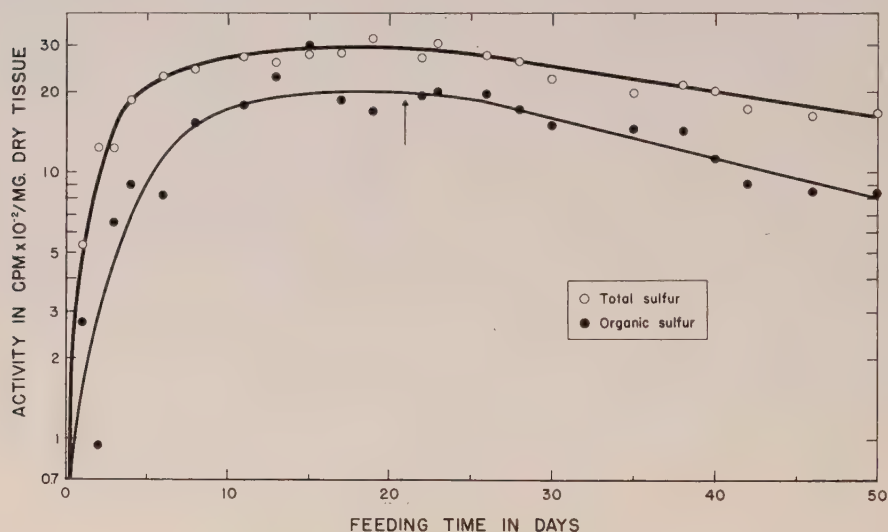


FIGURE 1. Radioactivity of sulfur observed in hydrolysates of male *Blattella germanica* (L.) fed $S^{35}O_4^{=}$.

per cent of absorbed $SO_4^{=}$ into an organic form. The arrow intersecting the curve for observed organic activity at the twenty-first day indicates the point in time at which the sources of $H_2S^{35}O_4$ were removed from the insect cages. The portions of the curve lying beyond this point represent the levels of activity remaining in the insects despite the normal catabolic activities over given periods of time. It is possible to estimate, even on the basis of these preliminary data, that the biological half-life of organic sulfur lies in the region of 21 to 22 days for this insect. It will also be noted that the activity of the total sulfur fraction decreased at a somewhat lower rate than did that of the organic fraction. This may be due to the degradation of the organic sulfur to form $SO_4^{=}$ in the insect tissues during the processes of catabolism. This shift of organic sulfur to the inorganic form coupled

with the normal direct excretory loss of organic sulfur may thus cause the organic activity to diminish at a faster rate than that of the inorganic fraction. Certain irregularities appeared from time to time in the data from which the curves were derived. For the present these must be considered to be the results of uncontrollable variations in the metabolic state of the test animals. As new information is obtained concerning limiting factors as yet unrecognized, it should be possible to eliminate such variations from the over-all experimental error.

STUDIES OF ANALYTICAL LOSS

A summary of the results of numerous studies of the losses of cystine and methionine in hydrolysis has been made by Block and Bolling (5). Under different conditions of acid hydrolysis the degree of destruction of cystine and methionine varies widely (1, 8, 19, 22, 27); losses of cystine up to 30 per cent and of methionine up to 20 per cent are commonly encountered.

The first test, carried out in quintuplicate, indicates that the precipitation of $\text{SO}_4^{=}$ by the techniques described above is highly efficient; the recovery of $\text{SO}_4^{=}$ due to carryover in the precipitation is limited to 2.4 per cent. These results agree essentially with those obtained by Machlin's group (21).

Subsequent studies in triplicate on the recovery of organic sulfur after precipitation of $\text{SO}_4^{=}$ with BaCl_2 show that recoveries should be very close to the theoretical values; the average recovery was 104 per cent. Precautions were taken to prevent even minute evaporational water losses in subsequent experiments.

When the recoveries of organic sulfur were checked after each of the various steps in the preparational procedure, it was found that in general the actual recoveries from such manipulations as evaporation to dryness, transferring from one container to another, and making dried samples up to known volume, were each reasonably close to the theoretical values, i.e., *ca.* 96 per cent. On the other hand, after acid or hot water hydrolysis (Table I), it was found that the hydrolysis and precipitation brought about average losses of 16 and 22 per cent respectively. The observed over-all recovery after all of the above treatments lay in the region of 62 to 63 per cent, and the net recovery for hydrolysis and precipitation was approximately 65 to 66 per cent.

The data in Table I relating to over-all recoveries from preparations hydrolyzed under various conditions indicate that neither differences in boiling chips, the use of formic-hydrochloric acid mixtures, nor additions of known amounts of carrier cystine and methionine markedly affected the end results. Recoveries of organic activity from preparations in water at room temperature for 15 to 16 hours were reasonably good (Table I,

Expt. No. 3); however, the recoveries of organic activity from preparations refluxed in boiling water for the same period of time were greatly reduced (Table I, Expt. Nos. 2 and 3). In fact, such recoveries agreed very closely with those obtained by refluxing duplicate preparations with 6 *N* HCl under identical conditions. It appears that the heating itself in the presence of water and air might be responsible for breakdown rather than the HCl present.

These losses described above are twofold in nature: the first is direct and

TABLE I
PERCENTAGE RECOVERY OF ORGANIC SULFUR AT VARIOUS STAGES IN THE PREPARATION OF RADIOASSAY SAMPLES UNDER DIFFERENT METHODS OF HYDROLYSIS

| Conditions of hydrolysis | Operational steps | | | | |
|---|-------------------|-------------|---------------|---------------------------|--------------------|
| | Hydrolysis | Evaporation | Precipitation | Decanting and evaporation | Over-all recovery* |
| Experiment #1 | | | | | |
| 6 <i>N</i> HCl, silicate chips | 81.4 | 106.0 | 81.0 | 97.7 | 68.2 |
| 6 <i>N</i> HCl, porcelain chips | 82.6 | 98.3 | 79.6 | 99.5 | 64.4 |
| Experiment #2 | | | | | |
| 6 <i>N</i> HCl, porcelain chips | 90.0 | — | 78.5 | 87.8 | 62.0 |
| Hot water, Teflon chips | 84.3 | — | 75.3 | 100.0 | 63.5 |
| 6 <i>N</i> HCl, 44% formic acid, Teflon chips | 86.2 | — | 92.4 | 98.0 | 78.0 |
| Experiment #3 | | | | | |
| Hot water, Teflon chips | 84.8 | — | 80.5 | 94.1 | 64.2 |
| Cold water, Teflon chips | 104.0 | — | 83.2 | 95.4 | 82.5 |
| Hot water, 50 mg. cystine, plus 50 mg. methionine, Teflon chips | 83.1 | — | 63.0 | 88.0 | 46.1 |

* Net recovery calculated from corresponding operational steps.

occurs during hydrolysis or heating itself. Since the total radioactivity was reduced, a certain amount of sulfur must have escaped the system completely; this could have been done only if the lost sulfur passed off as a gas. The second loss occurs during the precipitation of inorganic sulfur. The barium-sulfur reaction is quite specific; therefore it seems likely that the sulfur precipitated at that time existed in the form of $\text{SO}_4^{=}$. Since the original organic sample showed negligible inorganic activity on reprecipitation (2.4 per cent), the sulfur precipitated above must have been derived

from the organic sulfur of the sample. Moreover, the hydrolytic operation was the only chemical operation which had been carried out prior to precipitation; thus it must have been responsible for the production of $\text{SO}_4^{=}$ from organic sources.

For the present it should be noted that, while absolute losses occur, the relative relationships remain sound; variations tend to be self correcting in the curves shown. In the case of the chromatographic results, neither the differences in the degree of loss of cystine and methionine (estimated 10 per cent by Bailey, 1) nor the variations in loss are at all sufficient to account for the differences in specific activities cited below.

RESULTS OF CHROMATOGRAPHY

The results of chromatographing and radioassaying the organic sulfur fraction are given in Table II. The greater amounts of radioactivity were

TABLE II
 S^{35} ACTIVITIES OBTAINED FROM CHROMATOGRAMS OF *BLATTELLA GERMANICA* (L.)
HYDROLYSATES

| Strip | Strip width, Rf | Counts per minute | |
|-------|--------------------|-------------------|-------------|
| | | Total | Per Rf unit |
| X | 0.00-0.04 | 2008 | 502 |
| C | 0.04-0.13 | 9707 | 1078 |
| Y | 0.13-0.40 | 1868 | 69 |
| M | 0.40-0.56 | 6259 | 391 |
| Z | 0.56-1.00 | 0 | 0 |

found in the two strips of paper, C and M. Since these strips were based on the observed positions for cystine and methionine, it follows that the cystine and methionine contained detectable amounts of S^{35} . The X band of activity probably contains the activity due to the presence of traces of $\text{S}^{35}\text{O}_4^{=}$. The activity of the Y band is probably due both to the tailing of the cystine and the methionine on the paper and, perhaps, to the presence of unknown S^{35} -labeled products of metabolism. Apparently the major portion of the S^{35} found in the organic fraction of the hydrolysate is confined to cystine and methionine. Further evidence for this conclusion was found in an examination of the cystine and methionine isolated from the hydrolysate in the presence of relatively large amounts of cold carriers (Table III). Cystine and methionine so isolated showed a high degree of activity after this process of partial purification. Moreover, chromatographic analysis demonstrated that the cystine and methionine isolates were uncontaminated by other amino acids.

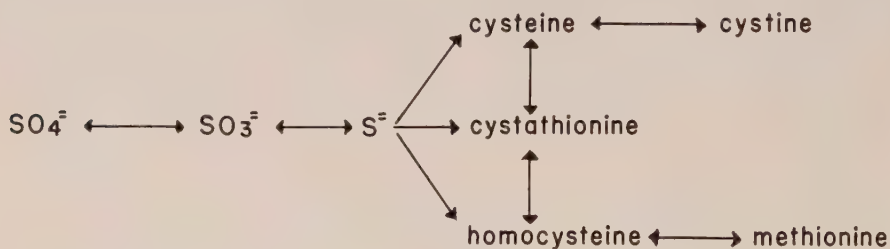
Evidence for the identification of the routes of synthesis of cystine and methionine may be obtained by examining the ratio of S^{35} -labeled cystine

TABLE III

RATIO OF SPECIFIC ACTIVITIES OF CYSTINE SULFUR AND METHIONINE SULFUR ISOLATED FROM *BLATTELLA GERMANICA* (L.) BY ISOTOPIC DILUTION OF VARIOUS HYDROLYSATES

| Source of sample | Specific activity in counts per minute per mg. | | | | Ratio of specific activity cystine sulfur/specific activity methionine sulfur |
|-----------------------|--|------------|----------------|-------------------|---|
| | Cystine | Methionine | Cystine sulfur | Methionine sulfur | |
| Nonaseptic adult male | 488 | 213 | 1,820 | 990 | 1.8 |
| Nonaseptic nymphs | 73,300 | 37,000 | 274,000 | 172,000 | 1.6 |
| Aseptic nymphs | 25,200 | 4,710 | 94,000 | 21,900 | 4.5 |

to methionine found in the cockroach hydrolysates (Table III). The cystine and methionine isolates were obtained from equal volumes of hydrolysate and in the presence of equal amounts of carrier cystine or methionine. Therefore, the ratio of the activity of cystine sulfur to methionine sulfur in the isolates is equal to the ratio of the activities in the original hydrolysates. Block, Stekol, and Loosli (7) have proposed a generalized diagram of the possible routes of synthesis of cystine and methionine from sulfate sulfur; this has been slightly modified for the purposes of this discussion:

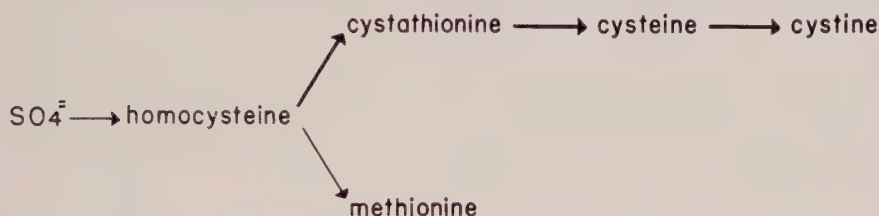


It is true that processes of degradation and excretion would modify this picture to some degree, but for the purposes of simplification such effects will be omitted.

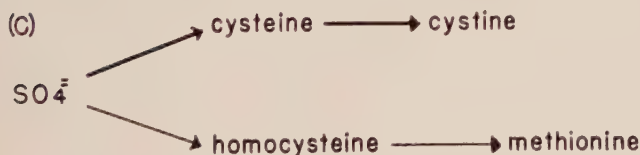
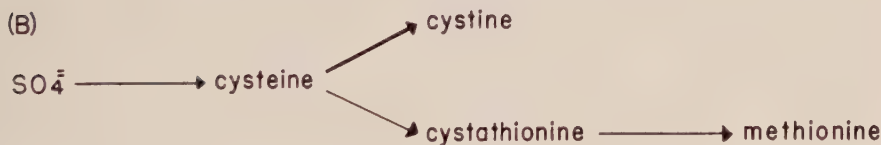
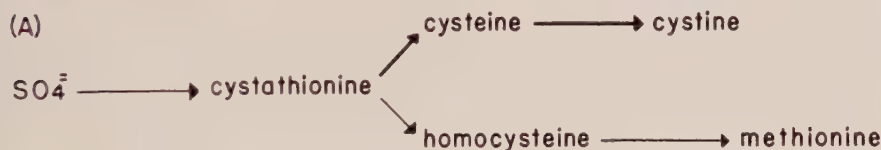
The observed ratio of cystine sulfur to methionine sulfur existing in the body of the male *Blattella germanica* (L.) under these conditions is approximately 0.83 (16), while the ratio of the specific activities is at least 1.6:1 for nonaseptic nymphs (Table III). The ratio is even higher in the case of the adult males (1.8:1) and aseptic nymphs (4.5:1). These data indicate that at least twice as much sulfate sulfur is incorporated into synthesized cystine as into methionine. In view of the fact that losses in organic activity due to hydrolysis have been observed it is necessary to note that the ratio of specific activities of cystine sulfur and methionine sulfur may have been affected by disproportionate losses of cystine and methionine (approximately 10 per cent). However, the determinations of the amounts of cystine and methionine sulfur were performed on the same

hydrolysates. Therefore, the losses of cystine and methionine would have affected each type of ratio equally.

While some cystine is possibly derived via transulfuration from homocysteine previously synthesized from $\text{SO}_4^{=}$ by the cockroach, its symbionts, or its commensals, a large portion of the cystine must be formed by some other mechanism. If all the sulfate incorporated into the organic fraction were to pass directly into the form of homocysteine, cystine could be produced via transulfuration as shown in the diagram below; the heavy arrows indicate the faster rate of synthesis:



This scheme would not seem logical since it presumes a faster rate of synthesis for cystine than for methionine, and this has not been observed in other species of animals. Therefore it is probable that cystine and methionine are produced by one of the routes described below:



Further quantitative study will be necessary to determine which one or which combination of these hypotheses is correct.

A question arises as to the reason for the difference between the specific activities of sulfur amino acids found in the adult males and the specific activities of those found in the nonaseptic nymphs. Dziewiatkowski (13) has found that young albino rats show greater ability to synthesize cystine from $\text{SO}_4^{=}$ than do older rats. Presumably the older rats or cockroaches tend toward a state of sulfur balance; thus the incorporation of sulfur would soon reach a rather low equilibrium value. On the other hand, the younger animals are in a state of positive nitrogen and sulfur balance; therefore, the sulfur is incorporated at a far higher rate and total amount in the younger animals.

It is also interesting to consider the cause of the change in the ratio between the specific activity of cystine and the specific activity of methionine when the nymphs were reared under aseptic conditions. There was a marked reduction in the synthesis of both of these compounds when aseptic conditions were imposed; the cystine activity dropped to 34.4 per cent of its nonaseptic level, while methionine activity was reduced to 12.7 per cent (Table III). Thus the ratio shifted from about 1.6:1 to approximately 4.5:1. Since the only known variable operating was asepsis, it may be assumed that the losses in activity and, therefore, the reduction in synthesis are due to the elimination of the gut microorganisms from the system. It should be noted that while these percentages must include the effects produced by the processes of interconversion and dissimilation of the sulfur compounds, these internal effects would presumably be similar in both contaminated and aseptic cockroaches. It follows that under nonaseptic, SH^- -deficient dietary conditions the intestinal microorganisms may be in large measure responsible for the production of both methionine and cystine in quantities sufficient to permit growth of this insect.

The growth of *Blattella germanica* (L.) may be suppressed by adding one per cent of succinyl sulfathiazole to a diet containing oxidized casein (deficient in methionine, cystine, and tryptophan) according to Noland and Baumann (24). They imply that suppression of those microorganisms in the gut that might synthesize methionine was responsible. Although the present study appears to confirm this idea, there is good reason to suspect that this chemotherapeutic agent may have also suppressed the normal metabolism of the cockroaches. De Groot has recently shown (11) that when succinyl sulfathiazole is fed to young adult bees at a level as low as 0.1 per cent in the normal diet, the growth of these insects, as evidenced by changes in their nitrogen content, was clearly inhibited. Since this compound has been shown to be a growth depressant for some insects, it is only reasonable to assume that succinyl sulfathiazole may not only interfere with fundamental processes in the microorganisms of the gut, but also with the intrinsic synthetic capacities of the cockroach, such as methionine synthesis, cystine formation, or perhaps the processes of transulfura-

tion. Moreover, the nutritional, metabolic, and toxic effects of the end products of methionine oxidation as found in oxidized casein have not been studied in detail for this insect. It is known that while methionine sulfoxide is utilized for growth in the albino rat, methionine sulfone, although nontoxic, is not thus utilized (2, 3). However, if a substance as toxic as methionine sulfoximine (14) were to occur in oxidized casein, its effect on the cockroaches might be rather pronounced.

A final complicating factor is the lack of information concerning the role of the intracellular symbionts. While they may play only a passive part in the sulfur metabolism, it is more likely that they exert at least some influence on the production of organic sulfur from inorganic precursors. In fact it is possible that the synthetic capacities of the aseptic cockroach may be largely due to their activities since external egg sterilization cannot assure their elimination from the system. It is difficult to draw concrete conclusions concerning the routes of production of organic sulfur compounds when such variables have not been identified and controlled. The effects of succinyl sulfathiazole and methionine oxidation products on the sulfur metabolism of contaminated and aseptic cockroaches should be studied by use of S^{35} . If suitable results are obtained, it will then be possible to give a final estimate of the reliability of insect nutritional techniques based on the use of chemotherapeutic agents.

Despite the probability that the intestinal microorganisms participate in the synthesis of sulfur-bearing compounds, the nonaseptic test insects showed no signs of being able to exhibit a typical rumen effect. If they were to do so, the symbionts, intracellular and otherwise, would produce all essential nutrients in amounts sufficient for normal growth and development. The cockroach, like the cow, could be made independent of external sources of all amino acids, vitamins, lipids, etc. It has been shown (10, 15, 18, 23, 24, 25) that this insect is not thus independent; moreover, it is able to carry on its activities in the absence of any gut commensals.

In some respects the performance of the septic cockroach differs markedly from that of both the albino rat and the laying hen. It is probably capable of synthesizing methionine as well as cystine; this is not true in the case of the hen which utilizes $SO_4^{=}$ for the production of egg cystine but not for egg methionine (20, 21) and the albino rat which produces cystine but no methionine (13). Moreover, it has been shown that while the rat and laying hen produce relatively small amounts of cystine from sulfate, this species of cockroach is capable of transforming at least 72 per cent of absorbed $SO_4^{=}$ into organic sulfur in the form of cystine and methionine.

No demonstration has been made of the role, if any, played by microorganisms of the digestive tract in the synthetic processes carried on in either the laying hen or the rat. While techniques of intramuscular injection were used for some of these studies, the intervention of gut organisms

has not been ruled out. The workers on these two animals suspect that the microflora of the gut may play a large part in the cystine synthetic capacities of these two species. Herein lies the major difference between these vertebrate species and the cockroach which not only is capable of synthesizing both cystine and methionine under aseptic conditions, but is able to carry this synthesis on at a rate great enough to permit survival and growth (15). It seems probable that such synthetic powers play a minor role in the metabolism of the cockroach when it is fed on diets which are optimal in their sulfur amino acid content. However, it is very likely that these abilities would become very important whenever these insects are forced to live on diets which are low in sulfur amino acids. It is possible that these faculties are intimately related to the capacity of the cockroach to survive under extremely adverse conditions.

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METABOLISM OF FUNGUS SPORES. I. OXIDATION AND ACCUMULATION OF ORGANIC ACIDS BY CONIDIA OF *NEUROSPORA SITOPHILA*¹

ROBERT G. OWENS

SUMMARY

Data reported in this paper show that conidia of *Neurospora sitophila* (Mont.) Shear & Dodge are capable of oxidizing α -ketoglutaric, succinic, fumaric, malic, oxalacetic and pyruvic acids or their products, which, in other organisms, play metabolic roles in Krebs's tricarboxylic acid cycle. All of these except oxalacetic acid were detected and provisionally identified by paper chromatography as naturally occurring substances in conidial homogenates. Three other spots which have not yet been identified were present also on chromatograms. Of the substrates tested glucose and acetate caused the largest acceleration of oxygen uptake. The endogenous respiratory quotient, which averaged .74, was shifted to unity or above by glucose but was diminished to an average of .27 by acetate. Therefore, gas exchange in conidia supplied with acetate was neither quantitatively nor qualitatively compatible with requirements for oxidation of acetate by way of the tricarboxylic acid cycle alone. Another pathway involving oxidation of the methyl carbon of acetate was suggested by an increase in glyoxylic acid upon addition of acetate to ungerminated conidia previously incubated for 20 hours under aerobic conditions. Such conidia showed increases also in the size of chromatographed spots corresponding to malic, fumaric, α -ketoglutaric, and one of the unidentified acids after 2 hours' incubation with acetate and in succinic acid after longer incubation. Increase in these acids was qualitatively in accord with the operation of a tricarboxylic acid cycle. Lactic acid was rapidly decarboxylated when supplied exogenously. Ethanol, the expected product of lactate decarboxylation, accelerated oxygen uptake. Both endogenous and exogenous oxidations were inhibited by $10^{-3}M$ cyanide.

INTRODUCTION

Fungus spores, like seeds of higher plants, are agents of dissemination and propagation of the species. As such, they are responsible for many plant diseases the world over. Prevention of spore germination and of subsequent parasitic establishment of the fungus on its host is fundamental to successful disease control by chemicals since most fungicides are preventative rather than eradicated in nature. Thus, the biochemical processes leading to germination of spores are the targets for chemical control of fungi and our knowledge of them needs to be extended for a more effective approach to the problem. Similarly, understanding of germination processes is fundamental to elucidation of the dynamics and biochemistry of transformations from static propagative organs such as

¹ Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

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spores and seeds to rapidly growing vegetative structures. Spore metabolism is thus of more than superficial importance in plant physiology and particularly in comparative plant biochemistry. Despite these important economic and academic ramifications, studies on spore metabolism have been relatively few. Where information is available, however, it testifies to the presence of a highly active metabolic machinery in spores capable of carrying out a great many hydrolytic and oxidative processes (6, 7, 8, 10, 13, 14, 16, 18, 19, 20, 21, 22, 27, 28, 33).

The present studies were undertaken in the hope of extending information on metabolism and enzymes in fungus spores and on the sequence of biochemical transformations during germination.

MATERIALS AND METHODS

Conidia of *Neurospora sitophila* were used throughout all tests. The fungus was cultured on Horowitz' medium containing glycerol (12) at laboratory temperature in 300-ml. Erlenmeyer flasks containing about 50 ml. of medium. Mycelium was permitted to develop for 2 days under sterile conditions. Flasks containing the vegetative fungus were then placed on a laboratory bench in the light and the cotton plugs removed to permit drying inside the flasks. With plugs removed abundant sporulation occurred overnight and spores of acceptably uniform age were obtained. If plugs were not removed mycelium usually filled the flasks in a few days but produced relatively few conidia. This was especially true when flasks were kept in a dark, closed place.

Gas exchange was measured by standard techniques at 30° C. (32) in Barcroft-Warburg respirometers. Contents of the flasks were agitated by operating the shaker at 100 strokes per minute. Tests on the effects of substrates and pH on oxygen uptake were conducted with quantities of spores varying from 20 to 120 million in 1.5 ml. of 1/15 *M* phosphate buffer. The sidearm contained 0.5 ml. of 4×10^{-2} *M* substrate, or water for controls, which was tipped into the main compartment of the flasks after equilibration of the systems in the water bath. Readings were begun after mixing the substrates and spores at time zero and were taken at 30-minute intervals over a period of 3 hours. Oxygen uptake by spores in the presence of each substrate and at each pH level was compared to that by equal volumes of spore suspension from the same preparation in systems which were identical except that water instead of substrate solution was added. These controls were used to calculate endogenous oxygen uptake. Gas exchange values in all tests were calculated on the basis of rates prevailing during the first 30 minutes of the tests, except as otherwise indicated in the legends of figures.

For tests in which respiratory quotients were determined, spores were

collected by centrifugation in a 15-ml. volumetric conical centrifuge tube, diluted with distilled water in the ratio of 1:7, and 1 ml. of this suspension (approximately 125 million spores) was used in unbuffered systems for determination of respiratory quotients. Substrate, 5.0 mg., was supplied in 0.5 ml. of distilled water. Control flasks containing water instead of substrate solution were always tested simultaneously. Systems were permitted to equilibrate in the water bath before mixing spores and substrates. Respiratory quotients were calculated from total gas exchange over a period of 1 hour, according to Umbreit, Burris and Stauffer (32). For CO₂ determinations, water was substituted for KOH in the center well, and gas exchange was compared with identical test samples in flasks containing KOH.

The identity of keto and other aliphatic acids in the conidia was determined provisionally by paper chromatography. Several grams of conidia were ground in a cold mortar with powdered glass. Glass and unbroken spores were removed by centrifugation. The supernatant solution was made up to 60 ml. and divided into two equal portions. One portion was deproteinized with tungstic acid solution and the keto acids converted to 2,4-dinitrophenylhydrazones and chromatographed as described by Cavallini and Frontali (3).

The remaining supernatant solution was treated with 5 grams (moist) of cation exchange resin (Amberlite IR 120) and filtered through Whatman No. 1 filter paper. The clear filtrate was evaporated to dryness at room temperature in an air current and the residue extracted with absolute ethanol. The ethanol was evaporated at room temperature. The residue was then taken up in a known volume of ethanol, usually 1 ml., and 5-10 λ of this, depending on the amount of residue obtained, was applied to Whatman No. 1 filter paper for development.

Two developing solutions described by Stark, Goodban and Owens (30) made up in the following proportions were used:

- 1) 5 ml. *n*-butyl alcohol, 5 ml. benzyl alcohol, 1 ml. of water and 0.11 ml. 90 per cent formic acid; and,
- 2) 1 ml. *tert*.-butyl alcohol, 3 ml. benzyl alcohol, 1 ml. isopropyl alcohol, 1 ml. of water and 0.12 ml. 90 per cent formic acid.

Data on substrate effects and respiratory quotients were evaluated by the analysis of variance. The level of significance is indicated in the table and legends of figures. Due to the wide range in unadjusted oxygen uptake values (see "Endogenous Respiration") in the determination of substrate effects, these values were converted to logarithms and significant differences expressed as percentages (17). In case of respiratory quotients actual oxygen uptake values were used in the calculations.

RESULTS

ENDOGENOUS RESPIRATION

When oxygen uptake by different numbers of conidia in the absence of additional substrates was converted to relative quantities of oxygen for 10^8 spores it was found that oxidative activity of different spore collections varied sometimes as much as 5-fold. In 12 tests made up of duplicated flasks oxygen uptake ranged from about 40 $\mu\text{l.}/\text{hr.}$ to 210 $\mu\text{l.}/\text{hr.}$ for 10^8 spores, with an average of 85 $\mu\text{l.}/\text{hr.}$, which is in accord with the findings of McCallan *et al.* (16) and Yoder (33). This extreme variability between tests made it impractical to compare tests with different spore collections on the basis of spore number and absolute quantities of oxygen consumed. However, the relative effects of substrates and pH were reasonably consistent. Therefore, the mean of endogenous oxygen uptake values in a given series of tests was determined. Oxygen uptake in the presence of the various substrates was then adjusted to correspond to the mean endogenous oxygen uptake value by use of the following relationship:

$$\frac{\text{Mean of endogenous O}_2 \text{ uptake values at pH } x \text{ for all tests}}{\text{Individual endogenous O}_2 \text{ uptake values at pH } x} \times \text{Individual O}_2 \text{ uptake value at pH } x \text{ in the presence of substrate} = \text{Adjusted exogenous O}_2 \text{ uptake value for the substrate}$$

where x is any pH value at which corresponding readings of oxygen uptake were made in the presence of added substrate and without added substrate. The oxygen uptake values thus adjusted in several tests were averaged to derive the points of pH curves given in the figures to follow. The relatively consistent differences between endogenous and exogenous oxygen uptake found in the present tests are in accord with those found by McCallan *et al.* (16).

Respiratory quotients (R.Q.) were reasonably consistent also for a given substrate and for endogenous respiration. In case of endogenous respiration the R.Q. varied ± 0.11 from a mean of .74. Therefore, R.Q. values and relative oxygen uptake based on equivalent endogenous oxygen uptake were used in comparisons dealing with different tests and substrates. These results will be presented in another section.

Variability between spore collections was apparently due to several factors but analyses of these are outside the scope of this report. It was determined, however, that time played a quantitative role in oxidative activities of spores suspended in water. Data plotted in Figure 1 A, which are a composite of endogenous oxygen uptake values obtained in 12 tests, show that endogenous oxidations determined at pH 6.0 reached a maximum after about 45 minutes and then declined in a more or less linear manner. After 3 hours, oxygen utilization had decreased 43 per cent. Other

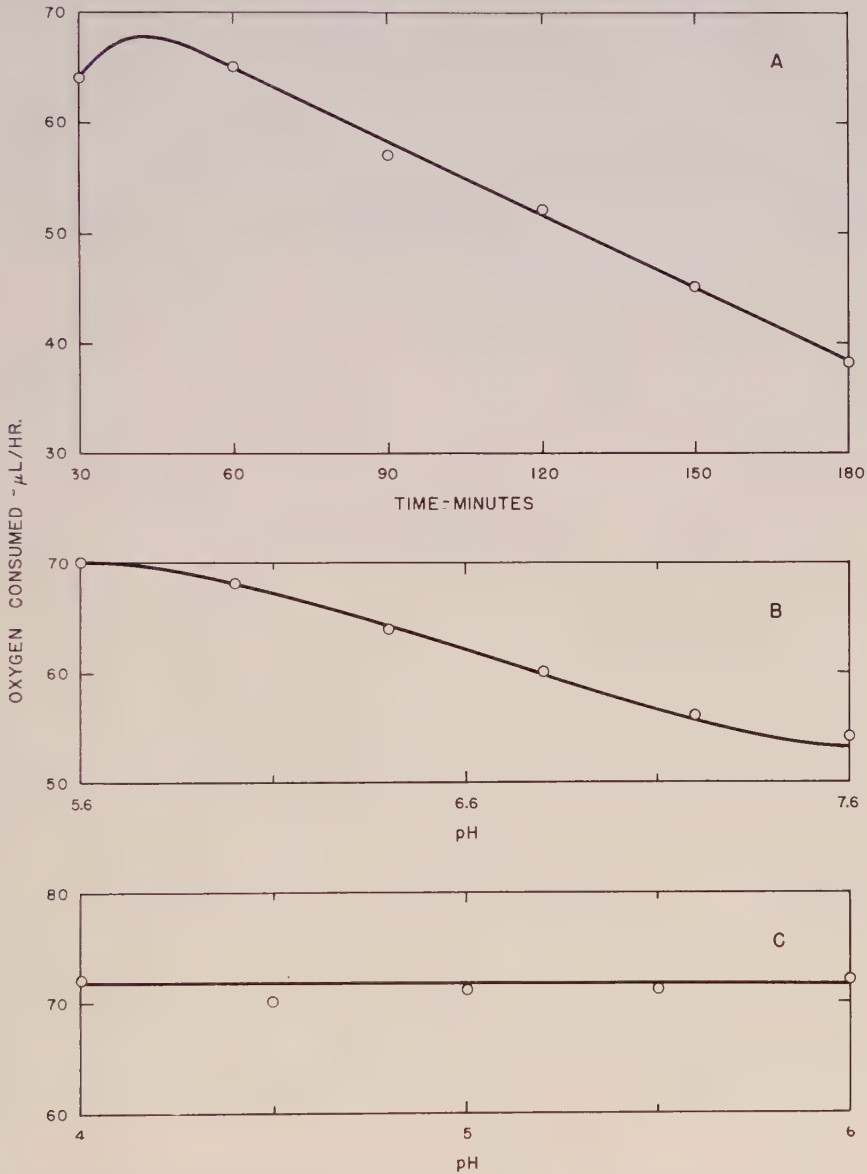


FIGURE 1. (A) Oxygen uptake by suspensions of *Neurospora sitophila* conidia in phosphate buffer at pH 6.0 over a 3-hour period. Curve is a composite average of 12 tests. (B) Oxygen uptake by suspensions of *Neurospora sitophila* conidia in buffers from pH 5.6 to 7.6. Buffers composed of Na_2HPO_4 and KH_2PO_4 . Curve is an average of 12 tests. (C) Oxygen uptake by suspensions of *Neurospora sitophila* conidia in buffers from pH 4.0 to 6.0. Buffers composed of Na_2HPO_4 and H_3PO_4 . Curve is an average of 2 tests.

tests showed, however, that the rate of decrease diminished between 3 and 9 hours so that some oxygen was still being consumed at the end of 9 hours. Conidia did not germinate during this period.

Endogenous oxidations were affected also by pH of the ambient solution buffered with phosphates (Fig. 1 B and C). Oxygen was consumed at the most rapid rate by conidia at pH 5.6 or below. The rate declined somewhat as pH of the solution was increased above 6.0. Between pH 5.6 and 7.6 it decreased an average of 23 per cent of the maximum, with a range of from 16 to 30 per cent. Between pH 4.0 and 5.6 uptake of oxygen appeared to be unaffected by pH.

The slight increase in endogenous oxygen uptake occurring as pH was changed from 7.0 to 6.0 suggested that pH effects might be concerned foremost with permeability of the spore membrane with regard to oxygen. Since this idea would be given credence if the internal pH of spores were

TABLE I
EFFECTS OF SUSPENDING CONIDIA OF *NEUROSPORA SITOPHILA* FOR 90 MINUTES
IN PHOSPHATE BUFFERS AT SEVERAL pH LEVELS ON pH OF THE
HOMOGENIZED CELL CONTENTS

| pH of buffer | pH of homogenate |
|---|------------------|
| No buffer | 6.35 |
| 4.0 | 6.75 |
| 4.5 | 7.00 |
| 5.0 | 7.05 |
| 6.0 | 7.10 |
| 7.0 | 7.15 |
| Distilled H ₂ O and powdered glass | 8.00 |

not changed in solutions of different hydrogen ion concentrations, tests on the effects of ambient buffers on internal pH were carried out. Spores were incubated 90 minutes in 0.1 *M* phosphates at pH 4.0 to 7.0. They were then sedimented and rinsed by repeated suspension and sedimentation in distilled water in a centrifuge until the pH of the supernatant solution reached a constant value. This usually required about 5 washings over a period of 10 minutes. The spores were then homogenized in a cold mortar with powdered glass and the pH of the homogenate determined with a Beckman pH meter. The data, given in Table I, show only small changes in pH of cellular contents of spores, but these changes were consistently toward the alkaline end of the pH scale, even with spores held at pH 4.0. A pH value of 8.0, possibly due to alkalis leached from the powdered glass, was obtained for the blank without spores, but this does not invalidate the relative values for different buffers since all determinations were handled in the same way as the unbuffered spores whose homogenates had a pH of 6.2 to 6.4.

Shifts towards higher pH values were noted also in supernatant solu-

tions from respiring spores. This shift was augmented when sodium acetate or other sodium salts were supplied to conidia. Since acetate is readily utilized by the conidia, it is conceivable that the increase in pH values may have been due to binding of the anion by the cell contents and formation of a free base from the cation and water. An analogous situation seems possible also in case of buffers containing sodium or potassium phosphates and would account for the observed rise in pH.

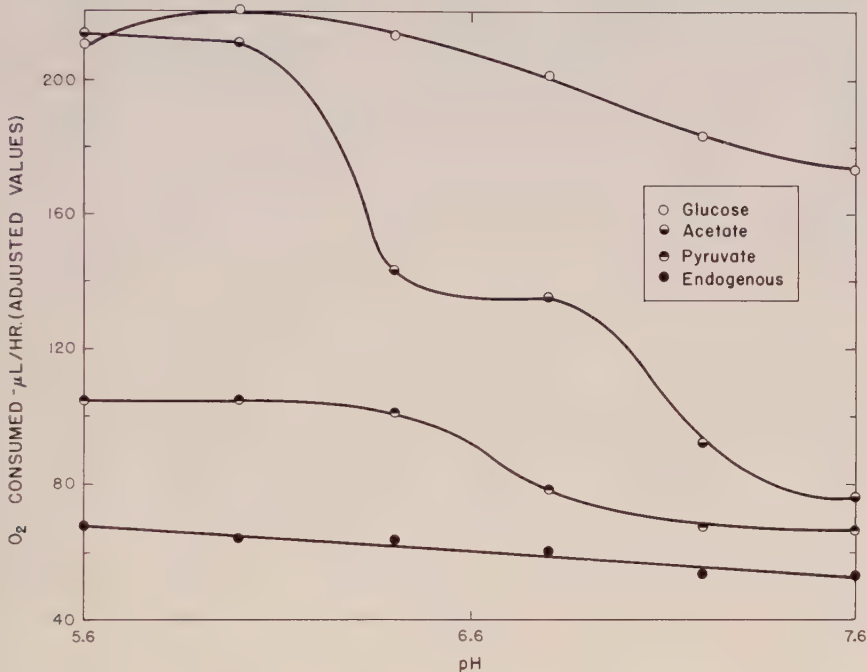


FIGURE 2. Oxygen uptake by suspensions of *Neurospora sitophila* conidia in the presence of 10^{-2} M acetate, pyruvate and glucose in phosphate buffers from pH 5.6 to pH 7.6. Buffers composed of Na_2HPO_4 and KH_2PO_4 . Differences of 15 per cent between endogenous points and points on the pyruvate curve are significant at the 5 per cent level. Differences of 30 per cent between endogenous points and points on the glucose and acetate curves are significant at the 1 per cent level of significance. Data adjusted as described in text.

EXOGENOUS RESPIRATION

Glucose, acetate, pyruvate and a number of tricarboxylic acid cycle intermediates were tested for their effects on the rate and maintenance of oxygen uptake by conidia. Results with glucose, acetate and pyruvate are shown in Figure 2, in which relative oxygen uptake, based on equivalent endogenous uptake, is plotted against initial pH of the solution. Analysis of the data shows that all substrates caused highly significant over-all in-

creases in oxygen uptake. Values for individual pH points in case of pyruvate are significantly different from endogenous values only at the 5 per cent level and only below pH 6.6. Acetate caused significant increases at pH values below 7.0 at the 1 per cent level, whereas all points on the glucose curve were significantly higher than endogenous oxygen uptake at the 1 per cent level. The difference required for significance for each point

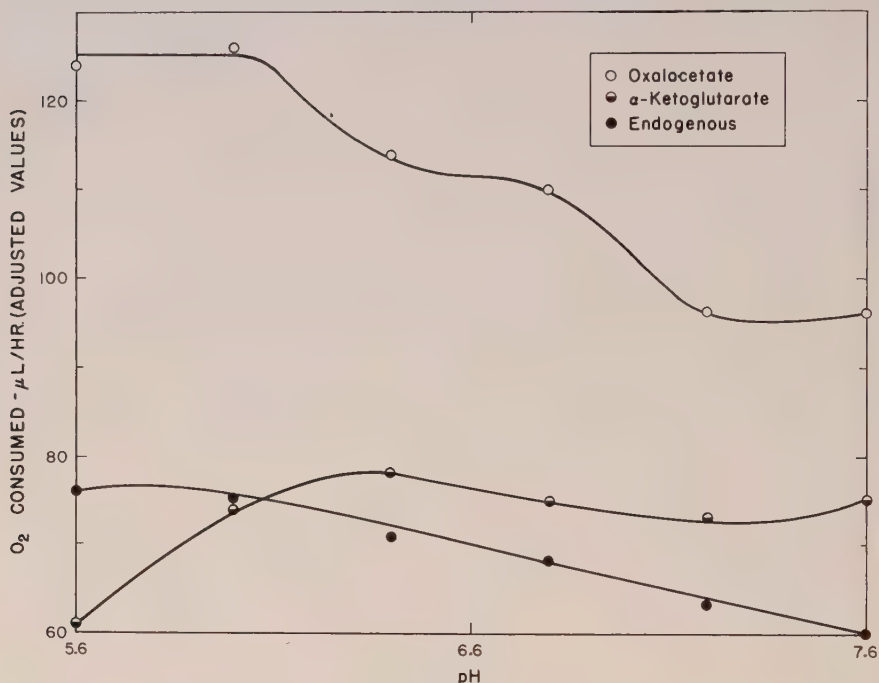


FIGURE 3. Oxygen uptake by suspensions of *Neurospora sitophila* conidia in the presence of 10^{-2} M oxalacetate and α -ketoglutarate in phosphate buffers from pH 5.6 to 7.6. Buffers composed of Na_2HPO_4 and KH_2PO_4 . Differences of 9 per cent between endogenous points and points on the α -ketoglutarate curve are significant at the 5 per cent level. Differences of 30 per cent between endogenous points and points on the oxalacetate curve are significant at the 1 per cent level. Data adjusted as described in text.

was 26 to 32 per cent at the 1 per cent level and 15 per cent in case of pyruvate at the 5 per cent level.

Distinct plateaus in pH profiles were obtained with these substrates. Slopes of the curves for the acids were consistently steeper than that for glucose. Since the former were supplied as sodium salts, pH of the medium drifted towards the alkaline end of the pH scale, whereas glucose caused a drift in the opposite direction. The oxygen uptake mechanism was adversely affected by increases in alkalinity so that the pH drift in the pres-

ence of sodium salts undoubtedly contributed to the decline in oxygen uptake rate with acetate and pyruvate as the pH was increased.

Similar drifts in oxygen uptake with change in pH were obtained with oxalacetate and α -ketoglutarate (Fig. 3). The oxalacetate profile was very similar in magnitude to that of pyruvate. All points on the curve were significantly different (at the 1 per cent level) from endogenous oxygen uptake. However, since the half life of oxalacetate in water is very short it is possible that this compound was decarboxylated and metabolized as pyruvate.

α -Ketoglutarate caused small increases in oxygen uptake. The over-all increase based on analysis of all points was significant at the 5 per cent level. However, individual uptake values between pH 6.0 and 7.2 were not significantly greater than endogenous uptake. The increase at pH 7.6 and the inhibition at pH 5.6 were significant.

Addition of sodium salts of non-keto members of the tricarboxylic acid cycle resulted in no significant increases in oxygen uptake in short term experiments. In order to detect differences due to added intermediates it was necessary to calculate total oxygen consumption over several hours. Since the pH was found to change over such extended periods, a true relationship between oxygen uptake and pH could not be obtained without elaborate equipment for controlling pH, which was not available, or the use of undesirably large quantities of buffer salts. Curves relating pH to the oxidation of these intermediates, therefore, were not obtained. Instead, oxygen uptake was measured at an initial pH of 3.0, which favors absorption (5, p. 362). The results are given in Figure 4. The rate of endogenous oxygen consumption at this pH was high and data presented in Table I would seem to indicate that the internal pH of the spores probably was not significantly altered. Data in Figure 1 C, although not covering pH values below 4.0, also suggest that endogenous oxygen uptake is probably constant in media considerably more acid than pH 4.0.

Statistically significant increases in oxygen uptake were obtained with succinic and fumaric acids after 3.5 hours and acceleration occurred within the first 20 minutes. Malic acid appeared to cause smaller increases beginning after about 1 hour but the accumulated oxygen uptake total after 3.5 hours was not significantly different from the endogenous total. Citric and isocitric acids likewise caused no significant increases in oxygen uptake.

It was noted that oxygen uptake in the presence of fumaric acid was essentially equal to that in the presence of succinic acid and considerably more rapid than that in the presence of malic acid. Since fumaric acid is not usually oxidized directly, but is first converted to malic or succinic acid or some other intermediate, it would appear that fumaric acid might be converted to succinic acid rather than to malic acid as would be ex-

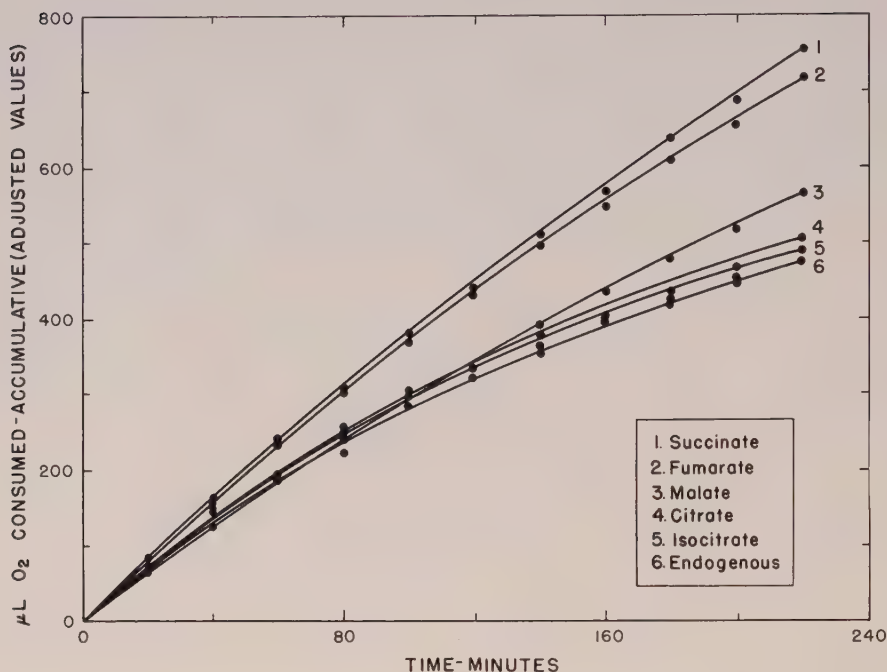


FIGURE 4. Oxygen uptake by suspensions of *Neurospora sitophila* conidia in the presence of succinic, fumaric, malic, isocitric and citric acids in phosphate buffer at pH 3.0. Buffer composed of Na_2HPO_4 and H_3PO_4 . Differences of 11 per cent between the final point on the endogenous curve and that on the substrate curves are significant at the 5 per cent level. Data adjusted as described in text.

pected if its utilization proceeded via the tricarboxylic acid cycle. However, the succinate-fumarate step is generally considered to be a reversible one and could occur in conidia if a source of hydrogen were available.

RESPIRATORY QUOTIENTS

Since the possibility of absorption difficulties made comparisons of oxygen consumption impractical as a criterion for establishing identities of oxidizable intermediates and pathways of oxidation, CO_2/O_2 ratios were determined for several substrates and compared with the theoretical requirements of the tricarboxylic acid cycle. The respiratory quotients obtained are given in Table II. The endogenous respiratory quotient (R.Q.), obtained as a mean of all controls, was $.74 \pm .11$, which is considered characteristic of fat metabolism. It was suspected that glycerol, which was the most abundant carbon source in the culture medium, might be the substrate responsible for the endogenous R.Q. As can be seen from data in Table II, although differences were not significant statistically, oxygen

TABLE II
EFFECTS OF SUBSTRATES ON OXYGEN UPTAKE, CARBON DIOXIDE RELEASE
AND RESPIRATORY QUOTIENT OF CONIDIA OF *NEUROSPORA SITOPHILA*†

| Substrate†† | Substrate-mean‡ | | R.Q. | Control-mean | | R.Q. | Difference | |
|---------------|-------------------------|--------------------------|--------|-------------------------|--------------------------|------|-------------------------|--------------------------|
| | μl. O ₂ /hr. | μl. CO ₂ /hr. | | μl. O ₂ /hr. | μl. CO ₂ /hr. | | μl. O ₂ /hr. | μl. CO ₂ /hr. |
| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (2)-(5) | (3)-(6) |
| Glycerol | 572 | 417 | .73 | 597 | 369 | .73 | 65 | 48 |
| Glucose | 606 | 727 | 1.05* | 478 | 323 | .68 | 218* | 404** |
| Acetate | 384 | 126 | .27 | 226 | 168 | .74 | 158** | -42* |
| Glyoxylate | 144 | 116 | .81 | 137 | 116 | .85 | 7 | 0 |
| Glycolate | 578 | 390 | .68 | 586 | 402 | .69 | -8 | -12 |
| Oxalic acid | 334 | 220 | .66 | 321 | 244 | .76 | 13 | -24 |
| Succinic acid | 445 | 265 | .58* | 356 | 256 | .72 | 89* | 9 |
| Malic acid | 201 | 195 | .97* | 182 | 124 | .68 | 19 | 71* |
| Pyruvate‡‡ | 184 | 137 | .74 | 183 | 147 | .80 | 1 | -10 |
| Lactic acid | 258 | 318 | 1.23** | 186 | 132 | .71 | 72* | 186** |
| Ethanol | 393 | 269 | .68 | 276 | 215 | .78 | 117** | 54* |

* Significantly different from the control at the 5 per cent level.

** Significantly different from the control at the 1 per cent level.

† Unbuffered conidial suspensions (approximately 125 million conidia).

‡‡ 5 mg. supplied in 0.5 ml. of water.

‡ Mean of 2 or more tests in duplicate.

‡‡ Pyruvate was not oxidized at a detectable rate under these conditions.

uptake and CO_2 release seemed to be increased by addition of glycerol but the R.Q. value remained equal to that for endogenous respiration. Theory would require 3.5 mols. of oxygen for oxidation of 1 mol. of glycerol, or an R.Q. of .86.

When glucose was added both oxygen uptake and CO_2 release were significantly accelerated, CO_2 release being more than doubled. R.Q. values averaging 1.05 were obtained, which is very close to the theoretical R.Q. value for carbohydrates. Acetate greatly increased oxygen uptake but apparently decreased CO_2 release with the result that the R.Q. was diminished to an average of .27. It thus becomes obvious that oxidation of acetate proceeded at a relatively much more rapid rate than decarboxylation. Theory, for oxidation of acetate via the tricarboxylic acid cycle, requires 2.0 mols. of oxygen for each 2 mols. of CO_2 released, or an R.Q. of 1.0.

The high rate of oxygen consumption in the presence of acetate thus indicated that some pathway of acetate oxidation other than the tricarboxylic acid cycle was operating. Some possible oxidation products, glycolate, glyoxylate and oxalate, were tested. None had any appreciable effect on the quantity of gas exchange or a significant effect on R.Q. values.

Another potential product of acetate oxidation, suggested by the oxidative condensation mechanism of Thunberg and Wieland (5, p. 400), is succinate. Succinate accelerated oxygen uptake but not CO_2 release resulting in an R.Q. of .58, which was significantly lower than the endogenous R.Q. For complete oxidation of succinate, theory requires an R.Q. of 1.14. Succinate, therefore, appeared to have an effect analogous to that of acetate. With either compound, products richer in oxygen or poorer in hydrogen than the substrate added would be expected to accumulate.

Malate supplied to conidia caused a significant increase in R.Q. from .68 for endogenous respiration to .97. Theory, for complete oxidation of malate, requires an R.Q. of 1.33. Malate, therefore, appeared to be oxidized, but, as was pointed out in the preceding section, it did not affect respiration in a way analogous to succinate, suggesting that they may be oxidized by different pathways.

Lactic acid, supplied to conidia, accelerated both oxygen uptake and CO_2 release and the R.Q. was found to be 1.23. The theoretical R.Q. for complete oxidation is 1.00. Thus, it would be expected that decarboxylation was at least partially nonoxidative in which case ethanol would probably be formed. Ethanol was found to accelerate oxygen consumption and CO_2 release significantly and resulted in an R.Q. of .68 which is the theoretical value for its complete oxidation.

PAPER CHROMATOGRAPHY

Data in the preceding section suggested that glucose was metabolized in the conventional way, i.e., by the Embden-Meyerhof-Parnas pathway and the tricarboxylic acid cycle, whereas acetate was apparently metabolized by some other pathway. To obtain another type of evidence, paper chromatography was used to identify provisionally the naturally occurring acids in conidia and some of the products accumulated upon oxidation of acetate. Several spots due to keto and other aliphatic acids from conidial homogenates occurred consistently on chromatograms. The

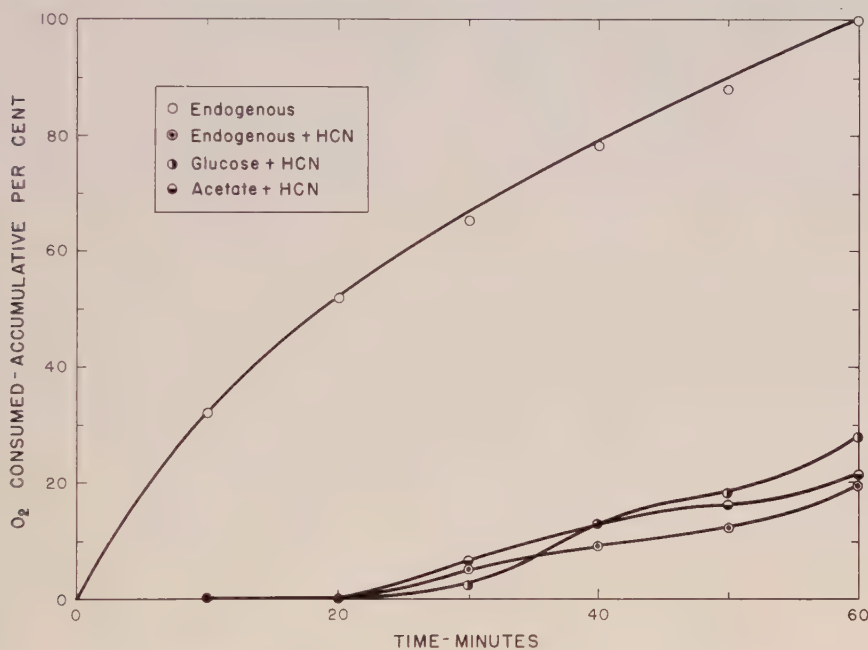


FIGURE 5. Inhibition of oxygen uptake by 10^{-3} M HCN.

average Rf values and lengths of spots, which are usually roughly proportional to quantity of material present, for known and unknown non-keto acids are given in Table III. Similar data are given also on acids obtained from conidial homogenates after incubation of conidia aerobically with 200 mg. of sodium acetate in order to obtain indications of the progressive change in size of spots with time.

The spots obtained for non-keto acids corresponded closely in position to malic, citric, succinic and fumaric acids. Two additional spots have not yet been identified.

Phenylhydrazones formed from naturally occurring keto acids corre-

TABLE III
CHROMATOGRAPHIC COMPARISON OF KNOWN ALIPHATIC ACIDS WITH ACIDS FROM HOMOGENATES OF *NEUROSPORA SITOPHILA* CONIDIA
BEFORE AND AFTER INCUBATION OF CONIDIA WITH ACETATE

| Solution No.* | Known† | | Acids from homogenates of spores incubated‡‡ | | | | | | | | | |
|------------------|----------|-----|--|----------------|----------------------------|----------------|------------------------|----------------|------------------------|----------------|-------------------------|----------------|
| | Acid | Rf | 0 hr. | | 20 hrs. without acetate | | 2 hrs. with acetate | | 6 hrs. with acetate | | 10 hrs. with acetate | |
| | | | Rf | Length, mm. | Rf | Length, mm. | Rf | Length, mm. | Rf | Length, mm. | Rf | Length, mm. |
| 1 | Succinic | .68 | .66 | 27 | .67 | 18 | .68 | 22 | .67 | 34 | .69 | 24 |
| | Fumaric | .75 | .75 | 25 | — | — | .73 | 22 | .73 | 16 | .75 | 20 |
| | Malic | .39 | .39 | 33 | .40 | 24 | .39 | 28 | .40 | 42 | .38 | 30 |
| | Citric | .32 | .28 | 25 | .31 | 26 | .30 | 30 | .31 | 28 | .29 | 28 |
| | Lactic | .63 | — | — | — | — | — | — | — | — | — | — |
| | Unknown | — | .03 | 13 | .03 | 16 | .03 | 12 | .03 | 14 | .03 | 14 |
| 2 | Unknown | — | .14 | 55 | .16 | 72 | .15 | 70 | .17 | 82 | .15 | 70 |
| | Succinic | .70 | .71 | 18 | .69 | 24 | .66 | 22 | .68 | 26 | .70 | 24 |
| | Fumaric | .77 | — | — | — | — | .74 | 24 | .74 | 18 | .76 | 18 |
| | Malic | .44 | .45 | 18 | .45 | 22 | .45 | 24 | .45 | 34 | .44 | 30 |
| | Citric | .36 | .36 | 25 | .38 | 26 | .37 | 32 | .37 | 28 | .35 | 30 |
| | Lactic | .65 | — | — | — | — | — | — | — | — | — | — |
| | Unknown | — | .07 | 12 | .07 | 16 | .06 | 14 | .06 | 18 | .06 | 14 |
| | Unknown | — | .23 | 52 | .19 | 63 | .20 | 72 | .21 | 76 | .20 | 72 |

* Solutions 1 and 2 described under "Materials and Methods."

† Average of 6 or more controls.

‡‡ Average of 2 or more spots at each time.

TABLE IV
CHROMATOGRAPHIC COMPARISON OF KNOWN 2,4-DINITROPHENYLHYDRAZONES OF KETO ACIDS WITH THOSE FORMED FROM ACIDS
IN HOMOGENATES OF *NEUROSPORA SITOPHILA* BEFORE AND AFTER INCUBATION OF CONIDIA WITH ACETATE

| Known† | Acids from homogenates of spores incubated†† | | | | | | | | | | | |
|-------------------------|--|-------------|-------------------------|-------------|---------------------|-------------|---------------------|-------------|----------------------|-------------|--------|-------------|
| | 0 hrs. | | 20 hrs. without acetate | | 2 hrs. with acetate | | 6 hrs. with acetate | | 10 hrs. with acetate | | | |
| | Rf | Length, mm. | Rf | Length, mm. | Rf | Length, mm. | Rf | Length, mm. | Rf | Length, mm. | Rf | Length, mm. |
| Phenylhydrazone | Isomer | Isomer | Isomer | Isomer | Isomer | Isomer | Isomer | Isomer | Isomer | Isomer | Isomer | Isomer |
| | 1 2 | 1 2 | 1 2 | 1 2 | 1 2 | 1 2 | 1 2 | 1 2 | 1 2 | 1 2 | 1 2 | 1 2 |
| Pyruvic | .54 .72 | .55 .72 | .52 .72 | 25 25 | .54 .72 | 23 16 | .57 .73 | 24 17 | .52 .72 | 25 17 | | |
| α -Ketoglutaric* | .24 — | .25 — | .25 — | 17 — | .26 — | 21 — | .23 — | 21 — | .25 — | 21 — | | |
| Glyoxylic | .44 .62 | .42 .62 | .43 .62 | 24 22 | .45 .62 | 29 21 | .45 .62 | 31 22 | .43 .62 | 29 27 | | |
| Oxalacetic | .26 .21 | — — | — — | — — | — — | — — | — — | — — | — — | — — | | |
| Unknown | — — | .79 — | .78 — | 10 — | .77 — | 15 — | .77 — | 19 — | .79 — | 15 — | | |

* One isomer only is formed.

† Average of 6 or more controls.

†† Average of 2 or more spots at each time.

sponded in position, number of isomers (3) and color upon spraying with 10 per cent KOH to glyoxylic, pyruvic and α -ketoglutaric acids. An unidentified spot with an average Rf of .78 was also present. These are given in Table IV.

Upon incubation of the spores for 20 hours on a shaker increases in all spots occurred. Upon incubation of the spores for additional periods of time in the presence of acetate apparent further increases occurred in spots corresponding to glyoxylic and α -ketoglutaric acids. Since these increases had already begun during the preconditioning period, further work of a quantitative nature is required to show whether or not acetate was directly responsible for the further increases. Other experiments of a preliminary nature indicate that the first product formed by conidial homogenates from acetate before oxidation is a compound which forms a phenylhydrazone but is not extractable from ethyl ether with sodium carbonate solution, and, therefore, is not a keto acid. Whether the reactions occurring in homogenates and in conidia are identical is as yet unknown.

INHIBITION OF OXIDATION

The effects of hydrogen cyanide on conidial respiration were determined at a concentration of 10^{-3} M HCN according to Umbreit, Burris and Stauffer (32). The results are given in Figure 5. Endogenous oxygen uptake and the oxidation of glucose and acetate were inhibited completely for 20 minutes.

DISCUSSION

Comparison of the data in the present study with those reported for mycelial metabolism (5, 15, 23) reveals many similarities. Foster (5, p. 411) has pointed out that direct evidence for Krebs's tricarboxylic acid cycle in fungi is lacking, but many, if not all, of the necessary intermediates in the cycle have been found in mycelium or in fungus secretions. Certain aspects of metabolism in conidia of *Neurospora sitophila*, such as the ability to oxidize some of the tricarboxylic acid cycle intermediates and the presence of these intermediates in conidia are compatible with requirements of the cycle. The cyclic nature of the interconversion, however, still remains to be proved. Data obtained on the oxidation of acetate by conidia would seem to indicate the presence of oxidative pathways capable of handling a greater volume of oxygen than was indicated for the tricarboxylic acid cycle intermediates. Other pathways were, in fact, suggested by the increase in glyoxylic acid when spores were incubated with acetate.

The data on R.Q. values obtained for acetate and succinate lead one to the conclusion that compounds richer in oxygen or poorer in hydrogen must accumulate in conidia supplied with these substrates. The accumulation of glyoxylic acid is in good accord with this conclusion and suggests that acetate and succinate might be precursors in the pathway leading to

glyoxylic acid formation. Reports of glyoxylic acid in fungi are relatively few and practically nothing has been proved about its derivation. Its metabolism and its role, if any, in synthetic processes are likewise unknown.

Although citric acid was not the most abundant acid in conidia, its presence may be significant in view of the presence also of other acids, which, in other organisms, play a part in the tricarboxylic cycle. The pathway of citrate formation in fungi has not been elucidated, but has been the target of several theories. As early as 1919 Raistrick and Clark (25) proposed that oxalacetic acid and acetic acid condensed to yield citric acid. Chrzaszcz and Tiukow (4) later postulated that citric acid was formed by oxidative condensation of malic and acetic acids. Bernhauer and Siebenäuger (2) speculated that succinic and acetic acids might be condensed to tricarballic acid, which in turn, might be oxidized to *cis*-aconitic acid which would yield citric acid upon hydration. Arguments for one or all of these mechanisms could be sustained on the basis of data obtained with *N. sitophila* conidia in the present study. In 1949, however, Stern and Ochoa (31) were able to synthesize citric acid enzymatically *in vitro* and to prove that it is formed by condensation of oxalacetic acid and "active acetate." The present tendency is to attribute citrate synthesis in all organisms to this mechanism. Inability to demonstrate oxalacetate in *N. sitophila* conidia would not exclude possibilities of its occurrence in the minute quantities required for formation of citrate by this mechanism.

The mechanism of synthesis of α -ketoglutarate, which is present in *N. sitophila* conidia, has not been proved in fungi and reports of its occurrence are relatively few. In the conventional scheme of citrate oxidation, α -ketoglutarate occurs as the decarboxylation product of oxalosuccinic acid. Although proof has not been obtained it is suspected that the unidentified spot at Rf .78 on chromatograms in the present studies is oxalosuccinic acid, since its Rf value is very close to that obtained by Cavallini and Frontali (3). Another possible route, and one which has been demonstrated in *Aspergillus oryzae* (5, p. 510) involves deamination of glutamic acid. This mechanism provides no indication, however, of the formation of glutamic acid in the first place, which is generally assumed to be via amination of α -ketoglutaric acid. In 1948 Ajl and Werkman (1) showed that succinic acid can be carboxylated via CO₂ fixation to yield α -ketoglutarate. If CO₂ were fixed by *N. sitophila* conidia the process occurred at an undetectable rate and in all probability the α -ketoglutaric acid decarboxylation would occur at a greater rate, since the rate of the latter process was measurable. Therefore, the best explanation for the presence of α -ketoglutaric acid in conidia at present seems to be its derivation from citric acid.

Synthesis of succinic and fumaric acids in fungi has been the subject of many studies (5, pp. 351-377). One of the more popular mechanisms

devised to explain their formation seems to be that of Thunberg and Wieland (5, p. 400) in which two molecules of acetate are oxidatively condensed to form succinic acid which, in turn, exists in equilibrium with fumaric acid. This type of condensation was seemingly given support by Slade and Werkman (29) who demonstrated that isotopic carbon in the carboxyl group of acetate, used as a metabolite, occurred in the carboxyl groups of succinate in some bacteria. In *N. sitophila* conidia, succinic and fumaric acids were oxidized and were also accumulated to some extent when acetate was supplied as a substrate. Although other intermediates of the cycle also seemed to increase, no evidence of the sequence of transformations was obtained. Further work on this subject is anticipated.

Many 2- and 3-carbon compounds have been identified in fungi and have been shown to be metabolized (5, 15, 23). Goepfert and Nord (9) showed that glycerol may be oxidized by *Fusaria* to the corresponding triose and presumably metabolized via the Embden-Meyerhof-Parnas pathway to pyruvic acid. In *N. sitophila* conidia the products of glycerol metabolism were not identified but pyruvate accumulated in conidia in considerable quantities when the fungus was cultured on glycerol as the principal carbon source, which suggests that the above pathway is operative.

Many fungi in the Mucoraceae have the ability to form lactic acid (5). Rotini *et al.* (26) have postulated that lactic acid is formed by decarboxylation of malic acid in fungi. Foster (5, p. 291), on the other hand, states that lactic acid originates by reduction of pyruvic acid. Recent studies by Ochoa *et al.* (24) show that there is an enzyme in some organisms which catalyzes decarboxylation of malic acid to lactic acid. Conidia of *N. sitophila* apparently do not accumulate lactate but metabolize it at a rapid rate. The high ratio of CO₂ evolution to oxygen consumed suggests that it is in part, at least, nonoxidatively decarboxylated. The expected product of decarboxylation is ethanol which is apparently completely oxidized to CO₂ and H₂O.

It is obvious that *N. sitophila* conidia are physiologically very active cells and that their metabolism is no less complicated than growing cells. Although proof of cyclic interconversions is lacking, potentialities for oxidation of certain tricarboxylic acid cycle intermediates are present. The rates of oxidation of some of these intermediates, however, would not seem high enough to account for the volume of oxygen consumed by conidia, so that other oxidative pathways might play a major role in metabolism leading to spore germination.

ACKNOWLEDGMENTS

The author wishes to acknowledge his indebtedness to Prof. Ray F. Dawson, Botany Department, Columbia University, for his suggestions

during the course of this work and for valuable criticisms and suggestions concerning the manuscript.

Thanks are due also to Dr. George L. McNew, Managing Director, Boyce Thompson Institute, for stimulation and encouragement in undertaking this problem, to Dr. S. E. A. McCallan for his services in statistical analyses of data, and to many other individuals at Boyce Thompson Institute for their suggestions and discussions on various phases of the problem.

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METABOLISM OF FUNGUS SPORES. II. CYTOCHROME OXIDASE, SUCCINOXIDASE AND PYRUVATE CARBOXYLASE SYSTEMS IN HOMOGENATES OF CONIDIA OF *NEUROSPORA SITOPHILA*¹

ROBERT G. OWENS

SUMMARY

A considerable portion of the endogenous respiration of *Neurospora sitophila* conidia was retained in conidial homogenates, but the R. Q. of .74 found previously for intact conidia was shifted to a value greater than 1 and the enzyme systems for oxidation of pyruvate, acetate and glucose were inactivated during the homogenizing process. However, evidence was obtained for the presence of active cytochrome oxidase, succinoxidase and pyruvate carboxylase systems in homogenates and it was found that addition of *p*-phenylenediamine under some conditions resulted in oxidation of pyruvate apparently by way of the cytochrome system. This was taken as an indication that inactivation of the system oxidizing pyruvate, acetate and glucose during the homogenizing process involved a part of the electron transport system other than the terminal system. Naturally occurring cofactors which take part in pyruvate oxidation in other organisms did not permit oxidation of pyruvate in homogenates.

INTRODUCTION

Differential absorption of materials from solution by fungus cells is known to influence the rate of utilization of these materials although they are present in excess in ambient solutions (3). This factor makes it impractical to compare the effects of different substrates on metabolism of whole cells on a quantitative basis in short term experiments. In an attempt to eliminate selective absorption through the spore membrane as a factor in metabolism of conidia of *Neurospora sitophila* (Mont.) Shear & Dodge and to study the intracellular distribution of enzymes, homogenates of conidia were prepared and tested for the oxidative activities which were demonstrated previously for intact conidia (8).

MATERIALS AND METHODS

Conidia of *Neurospora sitophila* grown and collected as described previously (8), were rinsed 3 times and cooled to 5° C. They were then homogenized in a cold mortar with powdered glass. Sucrose solutions up to 1 *M* and phosphate buffer, 1/15 *M*, of pH 5.0 to pH 7.6 were tried as homogenizing media. Since these solutions failed to yield homogenates

¹ Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

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capable of oxidizing glucose and acetate, conidia were also homogenized in the absence of sucrose and phosphates and no particular differences in activity were noted. The ratio of powdered glass to spores and liquid was always such that a stiff paste was obtained in the mortar; otherwise, with too much liquid, breakage of the spores was not accomplished without prolonged grinding. Grinding was continued for 5 minutes. A small volume of sucrose-buffer solution or water at 0° C. was mixed with the homogenate and powdered glass. Powdered glass and unbroken conidia were removed by centrifugation for 5 minutes at about 500 times gravity. The orange-colored supernatant solution was then used for gas exchange tests or kept cold and subjected to partitioning in an ultracentrifuge according to Goddard and Holden (4).

In gas exchange tests 2 ml. of homogenate were used in each Warburg flask. Additives such as coenzymes, 2 mg., were supplied to the homogenate in 0.5 ml. of water. Prospective substrates, 5 mg. in 0.5 ml. of H₂O, were added from the sidearm after equilibration in the water bath.

All manometry was done as described by Umbreit, Burris and Stauffer (10).

RESULTS

The oxidative activity of conidial homogenates supplied with several prospective substrates, which were previously shown (8) to be oxidized by intact conidia, is shown in Table I. The endogenous oxygen uptake of homogenates was much less than would be predicted for an equivalent amount of spores, and the capacity for oxidation of pyruvate, acetate and glucose without other additives was completely lost. Succinic acid, on the other hand, accelerated oxygen uptake in accord with previous findings.

Although oxygen uptake was not affected by pyruvate without additives, CO₂ release was markedly increased. Subsequent to this finding homogenates were partitioned in a high speed centrifuge and the supernatant and particulate fractions tested for pyruvate carboxylase activity.

TABLE I
OXYGEN UPTAKE BY HOMOGENATES OF CONIDIA OF *NEUROSPORA SITOPHILA*
SUPPLIED WITH SEVERAL PROSPECTIVE SUBSTRATES

| Substrate* | O ₂ consumed/hr., μ l. |
|-----------------|---------------------------------------|
| None—endogenous | 53 |
| Pyruvate | 55 |
| Acetate | 58 |
| Succinate | 77 |
| Glycerol | 57 |
| Glucose | 60 |

* 5 mg. of substrate in 0.5 ml. of water were supplied to 2 ml. of homogenate in phosphate buffer, pH 6.0, containing 10^{-3} M ATP, 10^{-2} M MgSO₄, and 2 mg. of cytochrome *c*.

TABLE II
DISTRIBUTION OF PYRUVATE CARBOXYLASE IN CONIDIA OF *NEUROSPORA SITOPHILA*

| Cell fraction | Substrate* | O ₂ consumed/hr., μl. | CO ₂ released/hr., μl. |
|---------------|------------|-------------------------------------|--------------------------------------|
| Homogenate | Pyruvate | 23 | 96 |
| | None | 7 | 8 |
| Supernatant | Pyruvate | 11 | 100 |
| | None | 11 | 8 |
| Particles | Pyruvate | 7 | 4 |
| | None | 6 | 6 |

* Including 1/15 M phosphate buffer, pH 6.0, 10⁻³ M ATP, 10⁻² M MgSO₄, and 10⁻² M pyruvate.

As shown in Table II, carboxylase activity was retained in the supernatant solution with none appearing in the particles. The activity of the supernatant solution was equal to that of the whole homogenate over a period of 1 hour, so that essentially all of the carboxylase was recovered after fractionation (Fig. 1).

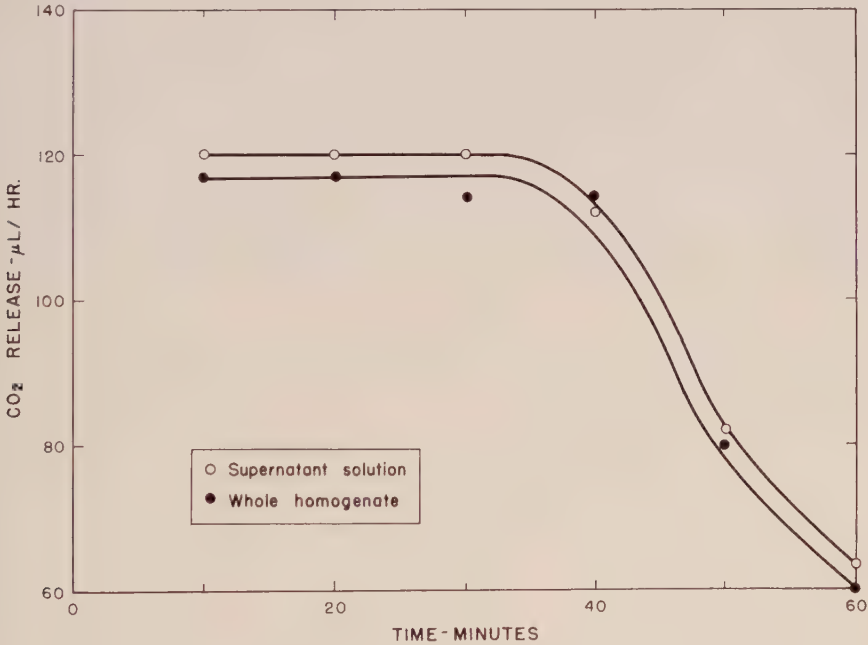


FIGURE 1. Course of activity of pyruvate carboxylase in whole homogenates of conidia of *Neurospora sitophila* and in the supernatant solution with cell particles removed by centrifugation at 10,000 times gravity.

In an effort to restore oxidative activity to homogenates in the presence of pyruvate a number of cofactors including the following were added: phosphates, Mg^{++} , Co A,² Co I and II, thioctic acid, malate, ATP, FAD, FMN, cytochrome *c*, indoleacetic acid, *p*-aminobenzoic acid, biotin, riboflavin, glutathione, thiamine, pyridoxal, nicotinamide, and cocarboxylase. None was effective alone or in several combinations. Since it was possible that apoenzymes instead of coenzymes were destroyed or inhibited in the homogenizing process, tests were carried out in an effort to locate the "block" in the homogenized system, beginning with cytochrome oxidase.

The presence and activity of cytochrome oxidase were tested by addition of *p*-phenylenediamine to homogenates, according to Keilin and Hartree (7) and others (6). Marked increases in oxygen uptake of homogenates in the presence of pyruvate and *p*-phenylenediamine occurred

TABLE III
EFFECTS OF *p*-PHENYLENEDIAMINE AND CYTOCHROME *c* ON ENDOGENOUS OXYGEN UPTAKE AND PYRUVATE OXIDATION IN HOMOGENATES OF CONIDIA OF *NEUROSPORA SITOPHILA*

| System* | O ₂ consumed/hr., μl. |
|--|-------------------------------------|
| Homogenate | 42 |
| Homogenate + pyruvate | 31 |
| Homogenate + pyruvate + <i>p</i> -phenylenediamine | 87 |
| Homogenate + pyruvate + <i>p</i> -phenylenediamine + cytochrome <i>c</i> | 156 |
| Pyruvate + <i>p</i> -phenylenediamine | 11 |
| Pyruvate + <i>p</i> -phenylenediamine + cytochrome <i>c</i> | 12 |
| <i>p</i> -Phenylenediamine + cytochrome <i>c</i> | 12 |
| Homogenate + <i>p</i> -phenylenediamine + cytochrome <i>c</i> | 20 |

* Each system was made up of 5 mg. pyruvate, 5 mg. *p*-phenylenediamine, 2.5 mg. cytochrome *c* in 0.5 ml. of water and 1 ml. of homogenate in phosphate buffer, pH 6.0. Where one component was excluded 0.5 ml. of water was substituted for it.

(Table III). Addition of cytochrome *c* resulted in additional increases, thus indicating the presence of active cytochrome oxidase (6, p. 126). Its presence was further indicated by increases in oxygen uptake caused by succinate (Table I), since the only mechanism known for oxidation of succinate is by way of the cytochrome system.

It was found, however, that if phosphates were excluded from the above system little or no increase in oxygen uptake due to pyruvate occurred. This suggests that the oxidation of pyruvate is a phosphorylative step even when coupled with *p*-phenylenediamine rather than with the naturally occurring hydrogen carrier. *p*-Phenylenediamine thus seemed to

² Abbreviations used here and subsequently are as follows: Co A = coenzyme A, Co I and II = coenzymes I and II, ATP = adenosine triphosphate, FAD = flavinadenine dinucleotide, FMN = flavinadenine mononucleotide.

provide a "shunt" pathway for transport of electrons from pyruvate to the cytochrome system.

Oxygen uptake by the "shunt" system was essentially constant over a period of 1 hour (Fig. 2). Carbon dioxide release, however, was very rapid and declined during the first 10 minutes when the remaining substrate was calculated to have reached about the same level as that after 30 to 40 minutes in other experiments (Fig. 1), suggesting that the decline was due to depletion of the pyruvate supply below a critical level required for

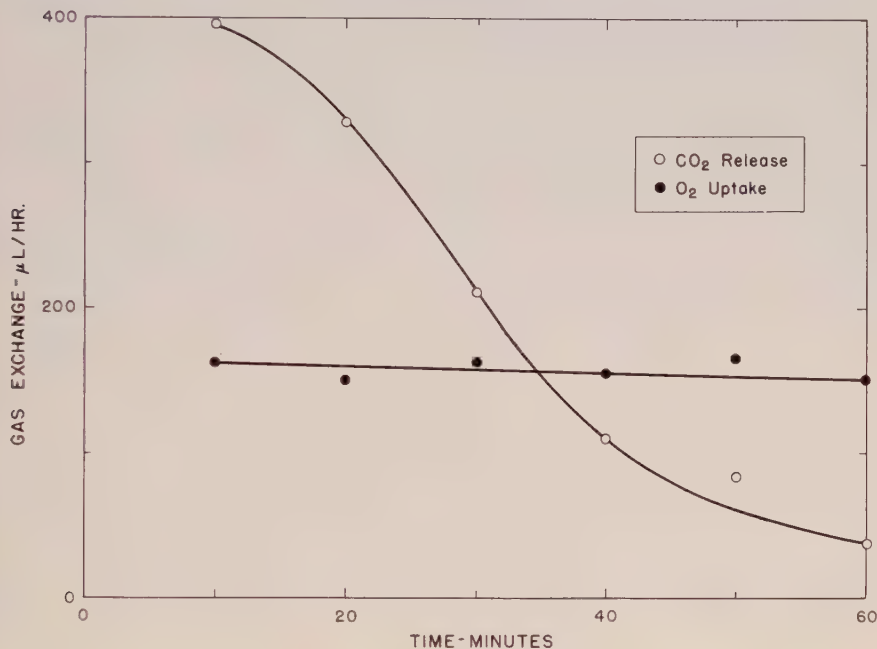


FIGURE 2. Course of oxidation and decarboxylation of pyruvate by homogenates of conidia of *Neurospora sitophila* in the presence of *p*-phenylenediamine and phosphates.

maximum CO₂ release. If this were true, the above data would indicate that maximal oxidation occurred at considerably lower concentration of pyruvate than maximal decarboxylation. The data show further that gas exchange was not stoicheometric at a given instance except in a special case where the two curves intersect after 36 minutes, but that the overall gas exchange might become stoicheometric after 80 minutes. This suggests that oxidation and decarboxylation were independent processes and that the product of pyruvate decarboxylation was the compound being oxidized.

Other data, given in Table IV, show that neither glucose nor acetate was oxidized by way of the phenylenediamine "shunt," and, further, sug-

TABLE IV

EFFECTS OF *p*-PHENYLENEDIAMINE AND CYTOCHROME *c* ON OXYGEN UPTAKE BY
HOMOGENATES OF CONIDIA OF *NEUROSPORA SITOPHILA* IN THE PRESENCE OF
GLUCOSE, ACETATE AND PYRUVATE

| Substrate* | O ₂ consumed/hr., μ l. |
|-----------------|---------------------------------------|
| None—endogenous | 55 |
| Pyruvate | 135 |
| Acetate | 58 |
| Glucose | 55 |

* 2.5 mg. of cytochrome *c*, 5 mg. of substrate, and 5 mg. of *p*-phenylenediamine in phosphate buffer, pH 6.0.

gest that neither is converted by homogenates to pyruvate or other possible substrates which may be oxidized by this process. Investigations on other possible products are being carried out.

Ferric and ferrous iron were also found to accelerate oxygen uptake by conidial homogenates. No additional increases in uptake were sustained, however, by the presence of pyruvate, acetate or glucose, as is shown in Table V. Iron, therefore, was unable to take the place of *p*-phenylenediamine in the pyruvate oxidation "shunt." The action of iron seemed to

TABLE V

EFFECTS OF Fe^{++} AND Fe^{+++} ON OXYGEN UPTAKE BY CONIDIAL HOMOGENATES
IN THE PRESENCE OF GLUCOSE, ACETATE AND PYRUVATE

| Exp. No. | Substrate* | O ₂ consumed/hr., μ l. |
|----------|------------------------|---------------------------------------|
| 1 | None | 15 |
| | Fe^{+++} | 23 |
| | Fe^{+++} and glucose | 23 |
| 2 | None | 39 |
| | Fe^{++} | 69 |
| | Fe^{++} and glucose | 74 |
| 3 | None | 66 |
| | Fe^{++} | 114 |
| | Fe^{++} and acetate | 128 |
| 4 | None | 10 |
| | Fe^{++} | 33 |
| | Fe^{++} and pyruvate | 25 |

* Substrates included 5 mg. of Fe^{+++} or Fe^{++} and 5 mg. acetate, pyruvate, or glucose.

be related to destruction of carotenoids present in *Neurospora* (5), since oxygen uptake due to added iron corresponded to the progress of decolorization of the homogenates.

DISCUSSION

Although the techniques employed in the present studies have not yielded homogenates capable of all oxidative activities carried on by intact conidia, no reasons were found to doubt that active oxidases can be obtained when appropriate conditions for preparation are found. The cytochrome oxidase and succinoxidase systems, which were obtained in active condition, appear to be no less active in homogenates than in conidia and to be sufficiently active to handle all of the oxygen consumed by intact conidia in the presence of the substrates tested.

The presence of these systems in *N. sitophila* conidia shows that certain basic similarities exist between conidia, mycelium, higher plants and animals with respect to terminal oxidase systems. Investigations by others (1, 2, 9) on spores and mycelium of this and other fungi lend support to this view.

The time relationship of O_2 and CO_2 exchange in the oxidation of pyruvate by way of the *p*-phenylenediamine "shunt" in conidial homogenates suggests that pyruvate is first decarboxylated and its product then oxidized, or, less likely, that the transfer of electrons to oxygen lags behind the release of CO_2 . The former possibility is compatible with present concepts of pyruvate decarboxylation with formation of acetaldehyde which may, in turn, be oxidized to acetic acid.

ACKNOWLEDGMENTS

The author acknowledges his indebtedness to Prof. Ray F. Dawson, Botany Department, Columbia University, for valuable suggestions during the course of these studies and for criticizing the manuscript.

Thanks are due also to Dr. George L. McNew, Managing Director, Boyce Thompson Institute, for his suggestions and encouragement on this phase of the problem.

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EFFECT OF LYOPHILIZATION ON THE VIABILITY OF LILIUM POLLEN

NORMA E. PFEIFFER

SUMMARY

Pollens of three species and three hybrids of *Lilium* were subjected to lyophilization for three hours with subsequent storage in evacuated tubes at -20°C ., 0.5° or 5°C ., and room temperature. When tested for viability after intervals of 50 to 194 days, the pollens gave low germination percentages immediately upon removal from storage.

With rehydration for five to seven days at 65 per cent relative humidity at 8°C ., there was increased germination. Longer exposure to these conditions resulted in a decrease in germinating capacity. However, transfer to -20°C . for two weeks maintained this lower germination percentage. Decline in germination ensued with a longer interval at -20°C .

Pollen of the same lots, not treated, but stored at similar low temperatures with relative humidity of 35 per cent or less, gave far better germination than lyophilized pollen. There is an indication that untreated pollen at room temperature with no humidity control may give less satisfactory germination than lyophilized pollen in evacuated tubes at the same temperature.

INTRODUCTION

The lyophil method has been found to be a successful technique for preserving the life of bacteria (7), fungi (5), and uredospores of *Puccinia* (6). Previous work on methods of storage has shown that *Lilium* pollen can endure temperatures below freezing as well as reduced atmospheric pressure (2, 3) and that deciduous fruit pollen and olive pollen can be successfully stored from one season to the next in the ordinary home freezer, at temperatures close to 0°F . (1). Tests were therefore undertaken to determine if lyophilization followed by storage at various temperatures would prove as effective for *Lilium* pollen as for lower organisms.

METHODS AND MATERIALS

In the relatively simple lyophilization apparatus used, phosphorus pentoxide served as dehydrant and dry ice covered with anhydrous ether gave the low temperature of -75°C . to -80°C . These were arranged after glass storage tubes containing *Lilium* pollen were placed in flasks connected with a vacuum pump. The pollen was then dried at reduced pressure at -75°C . to -80°C . for three hours and the storage tubes were removed and exhausted with the vacuum pump, sealed off and stored at -20°C . (food freezer), at either 0.5° or 5°C ., and at room temperature.

The pollens used in the preliminary tests were collected soon after dehiscence of the anthers in early afternoon of July 21, 1953, from flowers of

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1) a hybrid of *L. auratum* Lindl., 2) *L. auratum*, and 3) *L. henryi* Baker. The anthers spread out in clean dishes were dried in a cool dark place until the following morning, when the pollen was freed from stamen tissue and introduced into the storage tubes. Light cotton plugs were inserted above the pollen mass before lyophilizing.

The second series of tests was run with pollens variously handled before lyophilizing. *Lilium regale* Wils. stamens were collected before dehiscence, dried over anhydrous calcium chloride at room temperature for a short time, then overnight at 0.5° C. in a desiccator at 35 per cent relative humidity. Pollen of a hybrid of *L. Martagon* L. × *L. hansonii* Leicht. was held for a week after collection in the freezing compartment of an automatic refrigerator before being placed in the storage tubes. A smaller lot of pollen came from stamens of a hybrid of *Lilium auratum* growing in the greenhouse; these were air dried in a dark cool place, then held overnight in the same desiccator as *L. regale* pollen.

In both series, some pollen was diverted from each lot for storage without lyophilization, except for the *auratum* hybrid. The conditions used for comparison included -20° C. and room temperature without humidity control, 0.5° C. at 35 per cent relative humidity, and for *L. regale*, tubes exhausted of air with the vacuum pump, sealed and stored at the low temperatures.

Viability was tested by sowing pollen on hanging drops of a nutrient (sucrose) medium, with either 1 per cent agar or 1.5 per cent gelatin. To determine germination percentage duplicate drops were sowed in the afternoon, and counts were made the following morning of pollen tubes with a length at least three times the diameter of the pollen grain. The basis for each count was approximately 300 grains.

RESULTS

In the series of July 21, 1953, lyophilized pollen stored 50 to 55 days showed very low germination percentages when sowed immediately upon opening the evacuated tubes, regardless of the storage temperature. In an endeavor to rehydrate the pollen, the opened tubes were placed for five days at 65 per cent relative humidity at about 8° C. (An earlier attempt to rehydrate at room temperature had resulted in the development of mold.) After rehydration, the three kinds of pollen stored at -20° C. averaged 5, 18, and 20 per cent germination for duplicate drops, those stored at 5° C. gave 8, 20, and 11 per cent, and those stored at room temperature 0, 15, and 13 per cent. After a longer interval at 65 per cent relative humidity, averages for -20° C. storage were 9, 18, and 16 per cent, for 5° C. storage 17, 20, and 23 per cent, and for room temperature 0, 9, and 8 per cent. The failure of the first pollen (*L. auratum* hybrid) at room temperature was possibly due to a defective tube.

Wherever a second set of storage tubes was available, these lyophilized pollens were also tested after 194 days of storage. As in the earlier test, there was little or no pollen tube production with immediate sowing of pollen. With a rehydration interval of seven days, pollen stored at -20°C . averaged 8 per cent germination for the *auratum* hybrid and 18 per cent for *L. henryi*. On the other hand, untreated pollen stored at low temperatures, either with humidity control or with the very low relative humidity at -20°C ., retained viability to a greater degree than the lyophilized lots (Table I). This was also true of untreated pollen stored under reduced pressure.

Of the second series stored on June 29, 1954, *L. regale* pollen was in greatest supply, with average germination of 69 per cent at the beginning of the experiment. The *Martagon* hybrid pollen was older when subjected

TABLE I

PERCENTAGE OF GERMINATION OF LILIUM POLLEN ON ARTIFICIAL MEDIA AFTER
194 DAYS STORAGE UNDER VARIOUS CONDITIONS

| Pollen | Lyophilized-vacuum rehydrated 7 days | | Not lyophilized | | |
|--------------------------|---|-------------------------|-------------------------|------|---|
| | | | -20°C . | | $0.5^{\circ}\text{C.}/35\%$ R.H. Open |
| | 5°C . | -20°C . | Vacuum | Open | |
| <i>L. auratum</i> hybrid | — | 6 | 42 | 54 | 42 |
| | — | 10 | 44 | 55 | 62 |
| <i>L. auratum</i> | 6 | — | 48 | 61 | 52 |
| | 7 | — | 78 | 71 | 53 |
| <i>L. henryi</i> | 12 | 18 | 36 | 70 | 53 |
| | 26 | 18 | 58 | 78 | 54 |

to lyophilization, with a lower initial germination of 35 per cent. The *auratum* hybrid pollen, in short supply, had an original germination of 60 per cent.

Lyophilized pollen of *L. regale* stored at the different temperatures for 71 days gave a low percentage of germination upon being sowed immediately after opening the storage tubes, but an increase in germination with rehydration at 65 per cent relative humidity at 8°C . for five days (Table II). Here the germination percentages appeared to be in the same general range without regard to the storage temperature. This increased rate was not necessarily maintained with further exposure to the high humidity. However, removal of the rehydrated pollens to -20°C . maintained viability for at least a two-week interval with average germination percentages of 20, 27, and 18 per cent for pollens originally stored at -20°C ., 0.5°C . and room temperature. There was decline with longer storage as

seen in the results after a total of 87 days at -20° C. (about six months storage after lyophilization). Untreated pollen showed better germination after storage at low temperatures than did lyophilized pollen. After 161 days, an average of 68 per cent germination was obtained for pollen stored in open vials at -20° C. and 65 per cent for that stored at 0.5° C. with 35 per cent relative humidity. However, after storage at room temperature and rehydration of both lots, the lyophilized pollen gave higher germina-

TABLE II

PERCENTAGE OF GERMINATION OF LILIU REGALE POLLEN ON ARTIFICIAL MEDIA
AFTER 71 DAYS STORAGE UNDER VARIOUS CONDITIONS

| Treatment | Storage | | 71 days | 5 days re- hydrat. | 12 days re- hydrat. | +14 days -20° C. | +87 days -20° C. |
|-----------------|------------------|----------------|----------|--------------------------|---------------------------|---------------------------------|---------------------------------|
| | Temp. | Condi- tion | | | | | |
| Lyophilized | -20° C. | Vacuum | 6 8 | 36 39 | 21 21 | 19 21 | 6 9 |
| Not lyophilized | -20° C. | Vacuum | 81 82 | Not rehydrated | | | 53 56 |
| | | Open | 83 85 | Not rehydrated | | | |
| Lyophilized | 0.5° C. | Vacuum | 16 16 | 35 41 | 22 30 | 26 28 | 15 16 |
| Not lyophilized | 0.5° C. | Vacuum | 79 79 | Not rehydrated | | | |
| | | 35% R.H. | 75 80 | Not rehydrated | | | |
| Lyophilized | Room temp. | Vacuum | 5 6 | 30 34 | 18 19 | 17 19 | 6 7 |
| Not lyophilized | Room temp. | Open | 6 9 | 11 13 | 1 1 | — — | 0 0 |

tion percentages than the non-lyophilized. There was of course the possibility of definite fluctuation of moisture content in the untreated pollen with no humidity control during storage, which may well be unfavorable to retention of ability to produce pollen tubes.

The lyophilized pollen of the *Martagon* hybrid did not germinate in most of the tests, except for that stored at -20° C. where germination averaged 33 per cent; no increase in germination resulted from rehydration. Non-lyophilized pollen stored in an open vial at -20° C. gave 27 per cent germination; stored at 0.5° C. it averaged 37 per cent when in evacuated tubes and 36 per cent when in an open vial. After storage at room temperature no germination was obtained for either lyophilized or non-lyophilized pollen.

Pollen of the *auratum* hybrid in this series was tested 84 days after treatment and storage, with no germination of lyophilized cells stored at room temperature and at -20°C ., but 7 per cent germination for pollen stored at 0.5°C . Rehydration for two days gave slight improvement, while rehydration for seven days gave 18 per cent germination for lyophilized pollen stored at -20°C . and 12 per cent after storage at 0.5°C . Negative results for room temperature storage were obtained even after rehydration, for unknown reasons. Germination of lyophilized pollen was lower than in non-lyophilized pollen stored under reduced pressure; the latter gave 45 per cent germination after storage at -20°C . and 42 per cent after storage at 0.5°C .

DISCUSSION

It is clear that with the technique used, lyophilization does not improve the keeping qualities of *Lilium* pollen at the lower storage temperatures, although it may give better results than non-treated pollen held at room temperature. It nevertheless seems remarkable that so large a cell, developed in the highest group of plants and not apt to be exposed to comparable conditions in nature, should have the capacity to withstand such drastic treatment in even a small percentage of the total number. Reproductive cells and organs, especially in lower orders, are apparently more resistant to extreme external conditions, as, at times, are resting structures like spores in ferns and seeds. But the pollen grain with a function to be performed relatively quickly is usually considered an active cell readily injured by extreme drying.

Not all pollens retain their viability in a vacuum, as *Lilium* pollen has been found to do (2). This pollen is not dry at the surface as are many others. Whether the oil associated with the pollen has any role in connection with its survival is not known. But changes in the color of the oil seem to be accompanied by loss of germinating power.

There is evidence that the very low germination percentages obtained upon sowing lyophilized pollen immediately after the storage tubes are opened is related to the low moisture content of the pollen grains. Rehydration for an interval of five to seven days at 65 per cent relative humidity at 8°C . usually increased the germination percentage. Rehydration was earlier found to be effective in restoring germinating power in gladiolus pollen, held overlong at room temperature (4). Variation in different tests is probably related to several different causes. One that must be recognized is variation in sowing. The freshness of the pollen when treated is undoubtedly also a factor. Least satisfactory results were obtained with the *Martagon* hybrid pollen which had been collected a week prior to lyophilizing. Still another factor in variable results may be the interval between removal from the lyophil apparatus and evacuating and sealing the tube,

preparatory to further storage at the designated temperature. Results might conceivably be more consistent if there were no shift in temperature or vacuum at this stage.

The lyophil method calls for apparatus and for extra time in preparing and drying the organs used. With such obviously poorer results for pollen than are obtained with lower forms like bacteria and fungi, it cannot compete with the simpler storage methods that involve no previous preparation. The germination percentages of non-lyophilized pollen stored under the conditions of controlled relative humidity with low temperatures or the extremely low temperature of the food freezer with its necessarily low humidity are such that the pollen could be expected to give satisfactory seed sets if used for pollination in combinations of compatible parents.

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INTERACTIONS BETWEEN VERNALIZATION AND PHOTOPERIOD IN SPINACH

A. J. VLITOS AND WERNER MEUDT

SUMMARY

Spinach seed (var. Nobel) were soaked in $\frac{1}{2}$ M KH_2PO_4 solutions and exposed to temperatures of 2°, 5°, 15°, and 25° C. for periods of two, four and eight weeks. At the expiration of each storage treatment at the respective temperatures, the seed were germinated in a composted sod soil in greenhouse flats. The resulting plants were grown under 18, 14, 12, 10 and 8-hour photoperiods in the greenhouse or in temperature-controlled light rooms. Seed vernalized at 2° and 5° C. resulted in plants which formed macroscopic flower buds or open flowers sooner or in higher percentages at all photoperiods above 8 hours in the greenhouse and at all photoperiods including 8 hours in temperature-controlled light rooms. The critical photoperiod necessary for flowering in this variety was lowered from 14 to 8 hours as a result of vernalization. As the length of the vernalization period was increased from two to eight weeks the acceleration of flowering at shorter photoperiods was more pronounced. Preliminary studies indicate that soaking of seed in phosphorylated sugar solutions for short periods prior to vernalization influences the flowering response of the mature plants grown under 18-hour photoperiods.

INTRODUCTION

The influence of temperature on the photoperiodic response of spinach (*Spinacia oleracea* L.) has been discussed by various authors (3, 4, 5, 6, 7). Knott (6) in his classic study with several varieties of spinach summarized the basic features of the interaction between temperature and daylength and its influences upon seedstalk development. Spinach plants grown under a 15-hour photoperiod while exposed to temperatures of 70° to 80° F., 60° to 70° F., and 50° to 60° F. began seedstalk elongation sooner at 60° to 70° F. than at the other two temperature ranges. If plants were held for one month at 40° to 50° F., under a 15-hour photoperiod, and were later moved to higher temperatures they developed seedstalks more rapidly than plants which were grown continuously at higher temperatures. Knott concluded that the temperatures prevailing at different stages of growth of spinach exert an important conditioning effect on subsequent floral initiation and elongation in response to photoperiod.

During the course of our studies on the influence of treatments of seed with chemicals on the photoperiodic response of spinach (var. Nobel) it was observed that vernalized seed (moist seed stored for one month at 2° C.), subsequently germinated in the greenhouse and grown under 18-hour photoperiods, resulted in plants which formed macroscopic flowers 15 days sooner than plants resulting from seed which were stored at 20° to 25° C. Plants, resulting from seed stored at 2° C. for one month, grown under 8-hour photoperiods exhibited elongation of the central axis, a phenomenon

not observed in plants grown from seed stored at 20° to 25° C. These observations suggested that vernalization of spinach seed might alter the photoperiodic responses of this crop. The following experiments describe the influence of vernalization on the photoperiodic behavior of spinach under varying daylength.

MATERIALS AND METHODS

VERNALIZATION AND PHOTOPERIODIC TREATMENTS

Spinach seed (var. Nobel) were placed in Petri dishes and were soaked in 5 ml. of $\frac{1}{5}$ M KH_2PO_4 so as to retard germination until the seed were planted in soil (2). The seed were stored at vernalization temperatures of 2° and 5° C. and at higher temperatures of 15° and 25° C. for periods of two, four, and eight weeks. At 25° C. it was necessary to moisten the seed periodically with sterile water to prevent drying. Four replicates of 50 seeds each were used per treatment. At the expiration of the respective storage times at 2°, 5°, 15°, and 25° C. the seed were planted in a composted sod soil in flats either in the greenhouse or in temperature-regulated light rooms. Greenhouse temperatures ranged from 24° to 30° C. during the day and 12° to 18° C. at night. Light room temperatures were 24° C. during the day and 16° C. at night. Twenty-five plants per replicate resulting from seed kept at 2°, 5°, 15°, and 25° C. were exposed to daylengths of 18, 14, 12, 10, and 8 hours in a randomized block design. In greenhouse experiments additional light was supplied by growing plants under 500-watt Mazda incandescent lamps. Short daylengths were provided by covering plants with aluminum foil shields. In the light rooms provided with mercury-arc lamps, all plants were grown continuously under the specified photoperiod and controlled temperature.

CHEMICAL SEED TREATMENTS

Several sugars and phosphorylated sugars were tested for their effect on floral initiation when applied to spinach (var. Nobel) seed. Fifty seed were soaked in 5 ml. of solutions of the test compound in Petri dishes for 24 hours. At the end of this period the seed were washed free of the test chemical with distilled water and were stored moist at 2° or 25° C. for one week before being planted in composted sod soil in either the greenhouse or light rooms. Twenty-five plants resulting from seed so treated were grown under 18-hour and 14-hour photoperiods.

EXPERIMENTAL RESULTS

EFFECT OF VERNALIZATION AND PHOTOPERIOD ON FLOWERING

Germination of seed stored for two weeks at the various temperatures and the subsequent emergence of seedlings from soil were recorded and the

results from a typical experiment are given in Figure 1. Germination during storage was most pronounced at 5° C. Slight germination occurred at 2° C. but none of the seed germinated at 25° C. However, emergence of seedlings from seed stored at 2° and 5° C. was better than that of seedlings from seed stored at 15° or 25° C. or from non-vernalized, dry seed kept at room temperature.

In greenhouse experiments 11 per cent of the plants resulting from seed vernalized for two weeks at 2° and 5° C. formed macroscopic flower buds

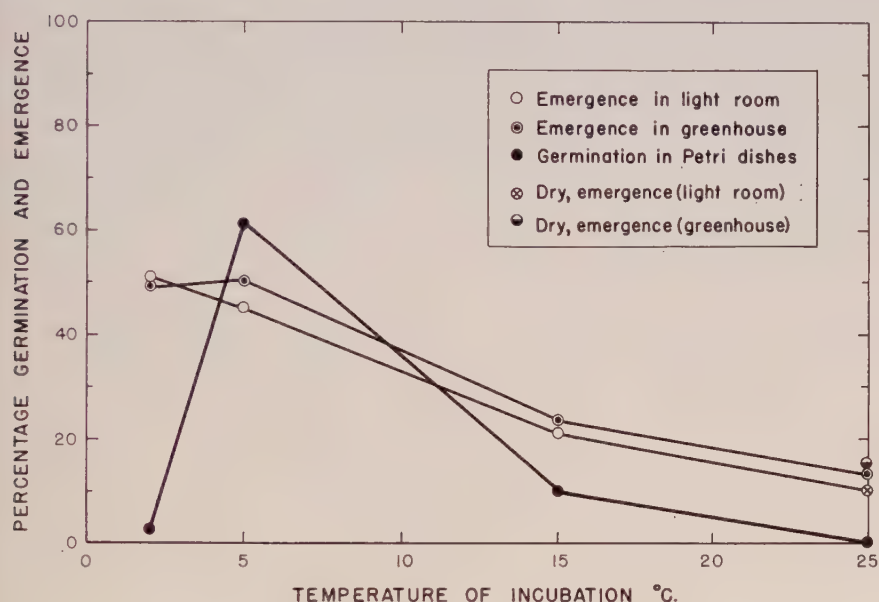


FIGURE 1. Germination and emergence of seed of spinach (var. Nobel) in Petri dishes and in greenhouse or light rooms after moist storage at 2°, 5°, 15°, and 25° C., and the emergence of seed in greenhouse or light rooms after dry storage at 25° C.

or open flowers eight days sooner under an 18-hour photoperiod than under shorter daylengths (Table I). Seed vernalized at 2° and 5° C. for four weeks resulted in approximately twice the number of plants with macroscopic flowers after 20 days under an 18-hour photoperiod as compared to a two-week vernalization period and a comparable photoperiodic treatment. A four-week vernalization period at 2° and 5° C. approximately doubled the percentage of plants with flowers after 28 days under a 14-hour photoperiod as compared to a two-week vernalization under a comparable photoperiod. As the critical photoperiod for flowering was lowered only those plants resulting from seed vernalized at 2° and 5° C. formed macroscopic flowers.

Under a 10-hour photoperiod, which is below the critical daylength for

flowering in this variety, only those plants resulting from vernalized seed exhibited the capacity to flower. When the vernalization period was increased from two to four weeks there was a marked increase in the percentage of plants with flowers. Although none of the plants flowered under an 8-hour photoperiod under greenhouse conditions, it was observed that several plants resulting from seed vernalized at 2° C. for four and eight weeks did exhibit stem elongation, a response related to seedstalk develop-

TABLE I
EFFECT OF VERNALIZATION ON THE FLOWERING OF SPINACH (VAR. NOBEL) GROWN UNDER VARIOUS PHOTOPERIODS IN THE GREENHOUSE

| Storage of seed | | Age of plants (days) | Percentage of 100 plants with macroscopic flowers under daylength of | | | | |
|-----------------|------------------|----------------------|--|---------|---------|---------|--------|
| Temp. (° C.) | Duration (weeks) | | 18 hrs. | 14 hrs. | 12 hrs. | 10 hrs. | 8 hrs. |
| 2 | 2 | 20 | 11 | 0 | 0 | 0 | 0 |
| | | 28 | 99 | 22 | 16 | 0 | 0 |
| | | 35 | 100 | 50 | 37 | 26 | 0 |
| | 4 | 20 | 28 | 0 | 0 | 0 | 0 |
| | | 28 | 88 | 50 | 25 | 32 | 0 |
| | | 35 | 100 | 81 | 33 | 33 | 0 |
| 5 | 2 | 20 | 16 | 0 | 0 | 0 | 0 |
| | | 28 | 94 | 32 | 9 | 0 | 0 |
| | | 35 | 100 | 32 | 35 | 25 | 0 |
| | 4 | 20 | 39 | 0 | 0 | 0 | 0 |
| | | 28 | 93 | 59 | 44 | 17 | 0 |
| | | 35 | 100 | 76 | 54 | 22 | 0 |
| 15 | 2 | 20 | 0 | 0 | 0 | 0 | 0 |
| | | 28 | 80 | 12 | 0 | 0 | 0 |
| | | 35 | 100 | 20 | 5 | 1 | 0 |
| | 4 | 20 | 0 | 0 | 0 | 0 | 0 |
| | | 28 | 66 | 13 | 0 | 0 | 0 |
| | | 35 | 100 | 27 | 0 | 0 | 0 |
| 25 | 2 | 20 | 0 | 0 | 0 | 0 | 0 |
| | | 28 | 67 | 8 | 0 | 0 | 0 |
| | | 35 | 100 | 9 | 4 | 0 | 0 |

ment in spinach. Non-vernalized controls were not observed to exhibit stem elongation under an 8-hour photoperiod.

The results of eight weeks vernalization and varying photoperiods on flowering under greenhouse conditions are shown in Table II. After 35 days 100 per cent of the plants resulting from seed vernalized at 2° C. and grown under a 12-hour photoperiod were flowering as compared with only 8 per cent for plants from seed kept at 25° C. for eight weeks. Similarly, under a 10-hour photoperiod 67 per cent of plants from vernalized seed were

TABLE II

EFFECT OF VERNALIZATION FOR EIGHT WEEKS ON THE FLOWERING OF SPINACH (VAR. NOBEL) GROWN UNDER SHORT PHOTOPERIODS IN THE GREENHOUSE

| Storage at ° C. | Age of plants (days) | Percentage of 100 plants with macroscopic flowers under daylength of | |
|-----------------|----------------------|--|---------|
| | | 12 hrs. | 10 hrs. |
| 2 | 20 | 25 | 0 |
| | 28 | 94 | 40 |
| | 35 | 100 | 67 |
| 25 | 20 | 0 | 0 |
| | 28 | 6 | 0 |
| | 35 | 8 | 0 |

flowering as compared to 0 per cent in plants from non-vernalized seed, including dry seed.

Results of similar experiments conducted in the light rooms are presented in Table III. Under these conditions the effect of vernalization upon flowering generally followed the pattern of the greenhouse experiments. However, there was a marked stimulation of floral development in plants from seed stored at 2° and 5° C. under the shorter daylengths of 10- and 8-hour photoperiods. In this case only those plants resulting from ver-

TABLE III

EFFECT OF VERNALIZATION FOR TWO WEEKS ON THE FLOWERING OF SPINACH (VAR. NOBEL) GROWN UNDER VARIOUS PHOTOPERIODS IN TEMPERATURE-CONTROLLED LIGHT ROOMS

| Storage at ° C. | Age of plants (days) | Percentage of 100 plants with macroscopic flowers under daylength of | | | | |
|-----------------|----------------------|--|---------|---------|---------|--------|
| | | 18 hrs. | 14 hrs. | 12 hrs. | 10 hrs. | 8 hrs. |
| 2 | 25 | 38 | 33 | 25 | 42 | 20 |
| | 29 | 72 | — | 38 | 48 | 24 |
| | 34 | 100 | — | 50 | 55 | 24 |
| | 46 | 100 | — | 55 | 55 | 24 |
| 5 | 25 | 32 | 7 | 17 | 11 | 10 |
| | 29 | 53 | — | 31 | 12 | 18 |
| | 34 | 100 | — | 42 | 20 | 18 |
| | 36 | 100 | — | 47 | 28 | 18 |
| 15 | 25 | 7 | 5 | 0 | 0 | 0 |
| | 29 | 20 | — | 0 | 0 | 0 |
| | 34 | 40 | — | 0 | 0 | 0 |
| | 36 | 100 | — | 0 | 0 | 0 |
| 25 | 25 | 6 | 0 | 0 | 0 | 0 |
| | 29 | 15 | — | 0 | 0 | 0 |
| | 34 | 28 | — | 0 | 0 | 0 |
| | 46 | 100 | — | 0 | 0 | 0 |

nalized seed flowered. Differences in response in the greenhouse and in the constant light room may have been due to light quality and intensity. This phase of the problem will be investigated further.

EFFECT OF CHEMICAL SEED TREATMENTS ON VERNALIZATION AND
PHOTOPERIODIC RESPONSES OF SPINACH

The effects of soaking seed for short periods in solutions of sucrose, glucose-1-phosphate, fructose-1,6-diphosphate, and acetone were studied

TABLE IV
INFLUENCE OF TREATING SEED WITH CHEMICALS AND VERNALIZATION ON THE
FLOWERING OF SPINACH (VAR. NOBEL)

| Seed soaked for 24 hrs. in solution | | 7-Day storage at ° C. | Percentage of 75 plants with mac- roscopic flowers grown under 18-hour photoperiods for | | | |
|--|-----------|-----------------------------|---|---------|---------|---------|
| Chemical | Concn., % | | 28 days | 32 days | 34 days | 36 days |
| Distilled water | — | 2 | 15 | 45 | 77 | 95 |
| | | 25 | 8 | 23 | 40 | 65 |
| Dry controls | — | 2 | 3 | 26 | 46 | 64 |
| | | 25 | 0 | 27 | 51 | 71 |
| Sucrose | 20 | 2 | 16 | 59 | 74 | 92 |
| | 10 | | 26 | 43 | 77 | 88 |
| | 5 | | 11 | 29 | 45 | 58 |
| | 1 | | 26 | 60 | 74 | 95 |
| Glucose-1-phosphate | 0.1 | 2 | 19 | 57 | 64 | 81 |
| | 0.01 | | 25 | 55 | 68 | 89 |
| | 0.001 | | 35 | 53 | 74 | 90 |
| | 0.1 | 25 | 3 | 32 | 39 | 63 |
| | 0.01 | | 13 | 48 | 53 | 69 |
| | 0.001 | | 5 | 33 | 58 | 57 |
| Fructose-1,6-diphosphate | 1.0 | 2 | 20 | 40 | 54 | 74 |
| | 0.1 | | 24 | 48 | 64 | 78 |
| | 0.01 | | 32 | 56 | 70 | 83 |
| | 1.0 | 25 | 0 | 26 | 59 | 71 |
| | 0.1 | | 13 | 33 | 64 | 72 |
| | 0.01 | | 8 | 57 | 58 | 87 |
| Acetone | 1.0 | 2 | 18 | 40 | 64 | 80 |
| | 0.1 | | 0 | 38 | 50 | 61 |
| | 0.01 | | 26 | 57 | 64 | 82 |
| | 1.0 | 25 | 0 | 38 | 50 | 61 |
| | 0.1 | | 5 | 42 | 57 | 69 |
| | 0.01 | | 7 | 30 | 53 | 85 |

in conjunction with the vernalization and photoperiodic responses of Nobel spinach. Fifty seed were soaked in Petri dishes in 5 ml. of aqueous solutions of sucrose (20, 10, 5, and 1 per cent concentrations), glucose-1-phosphate

(0.1, 0.01, and 0.001 per cent), fructose-1,6-diphosphate (1.0, 0.1, and 0.01 per cent) and acetone (1.0, 0.1, and 0.01 per cent) for 24 hours. Each treatment was replicated in triplicate. After 24 hours the seed were washed thoroughly with distilled water, divided in equal lots, and stored moist at 2° C. and 25° C. for one week. At the end of the storage period the seed were planted in greenhouse flats and twenty-five of the resultant plants per treatment were grown under 18-hour photoperiods. The percentages of plants with flowers were recorded after 28, 32, 34, and 36 days. The results are shown in Table IV. Those plants resulting from seed soaked in glucose-1-phosphate (0.001 per cent) or fructose-1,6-diphosphate (0.01 per cent) and stored for one week at 2° C. flowered sooner and in higher percentages than plants resulting from dry seed stored at 2° C. for one week or from seed soaked in water and receiving the 2° C. vernalization treatment. The only significant increase in macroscopic flowers was obtained with 0.001 per cent glucose-1-phosphate after 28 days. Similar results have been obtained in three successive tests under the same conditions.

DISCUSSION

The present experiments indicate that the flowering response of spinach (var. Nobel) is conditioned by an interaction between temperatures prevailing during moist seed storage and the subsequent length of day to which the seedlings and mature plant are exposed. It was possible therefore by exposing seed of this long-day variety to low temperatures for two weeks, to stimulate flower formation in mature plants grown under short-day conditions. The length of day became the limiting factor to flowering, however, if seed were not vernalized, or if plants from vernalized seed were grown under 8-hour daylengths in the greenhouse. It has been known for several years that although spinach is a long-day, annual plant, the temperatures prevailing during the time of development of seedlings exert an effect on the subsequent photoperiodic response of mature plants (6, 9). However, so far as the authors are aware this is the first report of seed vernalization in this crop.

Although the stimulation of flowering in spinach as a result of vernalization is undesirable from a practical standpoint, especially in areas where the crop is grown for table use, the interactions observed between low temperature and subsequent photoperiod may have fundamental value relative to photoinduction, dormancy, and related problems.

The biochemical changes which occur within seed and other storage organs as a result of exposure to low temperatures may suggest metabolic pathways leading to floral initiation. One of the most suggestive investigations of this type is the work of Arreguin-Lozano and Bonner (1) who studied the changes in sugars and sugar phosphates in potato tubers

stored at various temperatures. They found that sucrose accumulated in tubers stored at 0° C. and gradually decreased in concentration as the storage temperature was increased to 25° C. Fructose-6-phosphate and glucose-6-phosphate were also found to occur in greater amounts in tubers stored at lower temperatures. Miller (8) found that many treatments, including acetone, increased sucrose content in potato tubers. He was not able to find a close correlation, however, between increasing sucrose content and breaking of dormancy.

In our preliminary experiments 0.001 per cent glucose-1-phosphate applied to vernalized seed accelerated flowering when the resultant plants were grown under an 18-hour photoperiod, a favorable photoperiod for flowering in spinach (var. Nobel). However, under shorter photoperiods of 12 and 14 hours, where the effect of vernalization is most pronounced, it may be possible to accelerate floral development with chemicals which favor the formation of sugar intermediates in storage tissues. Experiments are presently under way to study the interaction between vernalization, seed treatments with phosphorylated sugars, and photoperiods below the critical daylength for flowering.

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USE OF FLUORESCENCE FOR THE ESTIMATION OF SUBSTANCES SEPARATED ON PAPER BY PARTITION CHROMATOGRAPHY

R. MAVRODINEANU, WILLIAM W. SANFORD, AND A. E. HITCHCOCK

SUMMARY

An adaptation is described of the Photovolt densitometer which makes possible the measurement of fluorescence from substances separated on paper by partition chromatography. The equipment consists of an ultraviolet light source, an ultraviolet transmitting glass filter followed by a filter transmitting only in the visible spectrum, and a photomultiplier tube connected to a measuring unit. Using this apparatus fluorescence measurements of indole-3-acetic acid (IAA) and an unknown substance separated from tomato plant extracts were studied. A proportionality between amount of substance per chromatograph spot and fluorescence was found and the lower limit of IAA estimation was determined as approximately 0.5 μ g. per spot.

Extracts of plant tissues frequently contain fluorescent substances of biological interest. The standard transmission densitometers are not suitable for the measurement of fluorescent substances which have been separated by paper partition chromatography. Such measurements are possible if certain changes are made in the densitometer. Through the cooperation of the Photovolt Corporation specified changes were incorporated into one of their densitometers which is now being used in our laboratory. This report describes the required changes in the instrument and its use for the rapid detection and estimation of known fluorescent substances and unknown fluorescent substances extracted from tomato tissue.

The densitometer made by the Photovolt Corporation (Standard Transmission Density Unit Model 52-C), together with the photomultiplier attachment (Multiplier Photometer Model 520-M), was chosen for conversion into a fluorescence-measuring system. The spectral sensitivity of the photomultiplier tube used has its maximum in the visible region. A high degree of sensitivity and flexibility is attained by the use of the photomultiplier with its amplifier which has a four-scale range ($\times 1$ to $\times 1000$). Setting $\times 1$ is used for greatest sensitivity and consequently, in general, for measuring relatively small amounts of a test substance. The fluorescence-exciting light source was provided by the mercury vapor lamp (General Electric 4-watt Germicidal lamp) delivered with the instrument. This mercury vapor lamp was inserted in place of the regular incandescent lamp, and an ultraviolet filter (U. G. 2, made by Schott, Jena) was inserted between the lamp and the aperture in a groove made under the aperture mount for this purpose.

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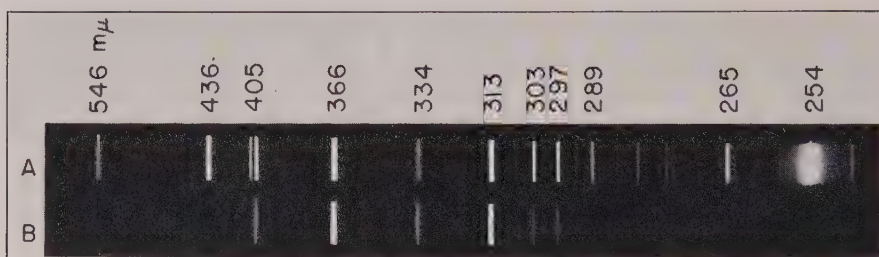


FIGURE 1. Spectrogram taken on a Kodak N 1 plate with a Hilger medium quartz spectrograph, using a slit aperture of 25μ and an exposure of 20 seconds. (A) Emission spectrum of the mercury vapor lamp used in the Photovolt densitometer. (B) Transmission of the U. G. 2 filter of 1 mm. thickness (Schott, Jena).

The spectral emission of the mercury vapor lamp is shown in Figure 1 A. The transmission of the filter using the same source of light is shown in Figure 1 B. This transmitted portion of the ultraviolet spectrum passes through the aperture (diam. 0.5 cm.), falls on the paper placed above the aperture, and reaches the photomultiplier tube of the search head after passing through a second filter placed in the filter turret of the search head.

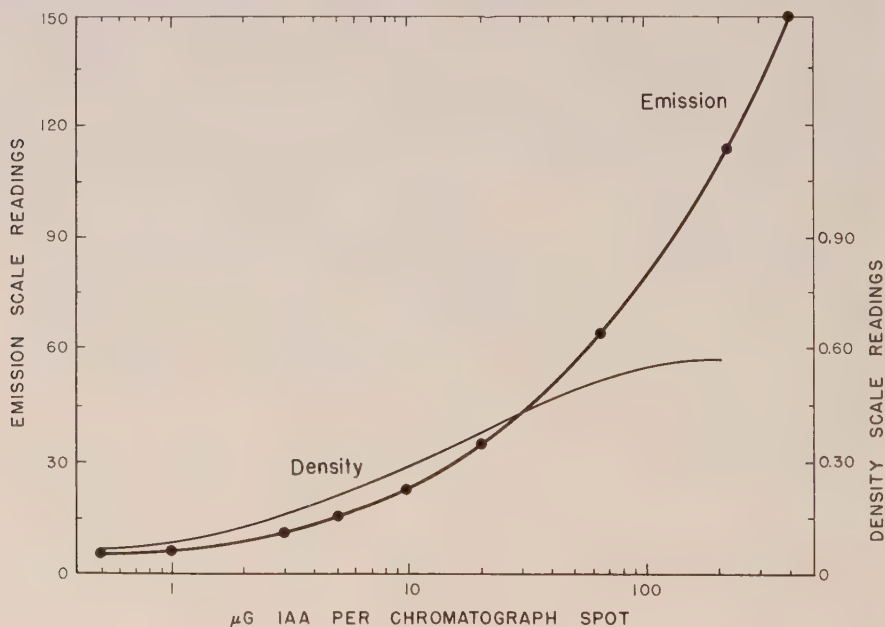


FIGURE 2. Emission and density readings for different quantities of IAA measured 30 to 45 minutes after removal of the paper from the developing tank. Emission readings involved the use of a $465\text{ m}\mu$ filter, an aperture of 0.5 cm., and a photomultiplier setting of $\times 1$. Corresponding conditions for density readings were $550\text{ m}\mu$, 0.5 cm., and $\times 1000$.

This filter eliminates most of the ultraviolet emission from the mercury vapor lamp and is transparent to the visible portion of the spectrum. For this purpose use was made of filters having a maximum transmission at 465, 495, 515, and 570 $m\mu$ which were provided with the instrument.

TABLE I
EMISSION OF CHROMATOGRAPHED INDOLE-3-ACETIC ACID UNDER
ULTRAVIOLET (297 TO 405 $M\mu$) RADIATION
(PHOTOMULTIPLIER SETTING $\times 1^*$)

| $\mu g.$ IAA per spot | Filter 515 $m\mu$ | Filter 495 $m\mu$ | Filter 465 $m\mu$ |
|-----------------------|----------------------|----------------------|------------------------------|
| 0.5 | | | 5.0 6.0 5.0 |
| 1.0 | | | 7.0 6.0 7.0 |
| 3.0 | | | 11 10 11 |
| 5.0 | | | 16 17 16 |
| 10 | | | 22 21 22 |
| 20 | 5.0 5.5 5.0 | 11 13 11 | 36 38 36 |
| 64 | 7.0 8.0 7.0 | 17 20 19 | 61 67 63 |
| 200 | 11 13 12 | 37 38 37 | 110* 120* 115* |
| 400 | 16 22 16 20 | 55 61 56 60 | 145* 160* 145* 150* |

* Readings obtained with photomultiplier setting $\times 10$ were converted by multiplication to values equivalent to setting $\times 1$.

Filter selection was made according to the fluorescence characteristics of the substances examined. This setup, in the absence of fluorescence, eliminates practically all the emission coming from the mercury vapor lamp, the instrument being zeroed under these conditions. In the presence of a fluorescent material on the paper, the emission excited by the ultra-

violet radiation passes through the conveniently selected second filter to the photomultiplier tube and produces a deflection on the microammeter proportional to the emission which, in a given range, is proportional to the amount of the material examined.

Known amounts of IAA, purchased from Eastman Kodak Co., were chromatographed by the ascending method, using a solvent of 80 parts isopropyl alcohol, 15 parts water, and 5 parts ammonia (28 to 30 per cent). The position of the IAA following 17 hours' development was determined either by treating a guide strip with 2 per cent *p*-dimethylaminobenzaldehyde in 1 *N* HCl or by examination under an ultraviolet lamp. Emission

TABLE II

EMISSION, UNDER ULTRAVIOLET (297 TO 405 $m\mu$) RADIATION, OF UNKNOWN MATERIAL (RF 0.25 TO 0.20) CHROMATOGRAPHICALLY SEPARATED FROM TOMATO PLANT EXTRACT (PHOTOMULTIPLIER SETTING $\times 10^*$)

| Grams of tissue per spot | Filter 570 $m\mu$ | Filter 515 $m\mu$ | Filter 465 $m\mu$ |
|--------------------------|-------------------|-------------------|-------------------|
| 1 | 0.95* | 7.0 | 16 |
| | 0.90* | 6.0 | 14 |
| | 0.95* | 7.0 | 15 |
| 3 | 3.6* | 18 | 48 |
| | 2.7* | 15 | 44 |
| | 3.1* | 18 | 48 |
| 4 | | 19 | |
| | | 26 | |
| | | 19 | |
| 5 | 5.6* | 27 | 83 |
| | 4.2* | 22 | 69 |
| | 5.3* | 27 | 83 |

* Readings obtained with photomultiplier setting $\times 1$ were converted by division to values equivalent to setting $\times 10$.

of the spots was then read as described above. Emission readings obtained with secondary filters 515, 495, and 465 $m\mu$ increased with increasing amounts of IAA on the chromatograph spot (Table I). Optimal readings shown in Figure 2 were obtained with the 465 $m\mu$ filter and measured at the settings $\times 1$ and $\times 10$ on the photomultiplier. In contrast, optical density at 550 $m\mu$ measurements were made at the least sensitive setting ($\times 1000$) and are included in Figure 2 in order to show the general shapes of emission and density curves. Any filter which transmitted more of the violet spectrum did not allow zeroing of the photometer except at the minimum sensitivity range, as too much of the ultraviolet light from the mercury lamp was admitted to the photomultiplier tube. With the 465 $m\mu$ filter and setting $\times 1$, the zero fluctuated plus or minus one division, thus establishing the lower limit of IAA estimation at approximately 0.5 $\mu g.$ per

chromatograph spot. The upper limits have not been established.

Following chromatography, as described for IAA, the estimated quantity of an unknown fluorescent substance in an ethanolic extract of tomato tissue was determined. A well-separated substance of moderate fluorescence (relative to other fluorescent spots) was selected at R_f 0.25 to 0.29 (IAA R_f 0.54). Dilutions of plant extracts representing 1 to 5 grams of tissue per spot were chromatographed. Emission readings for these

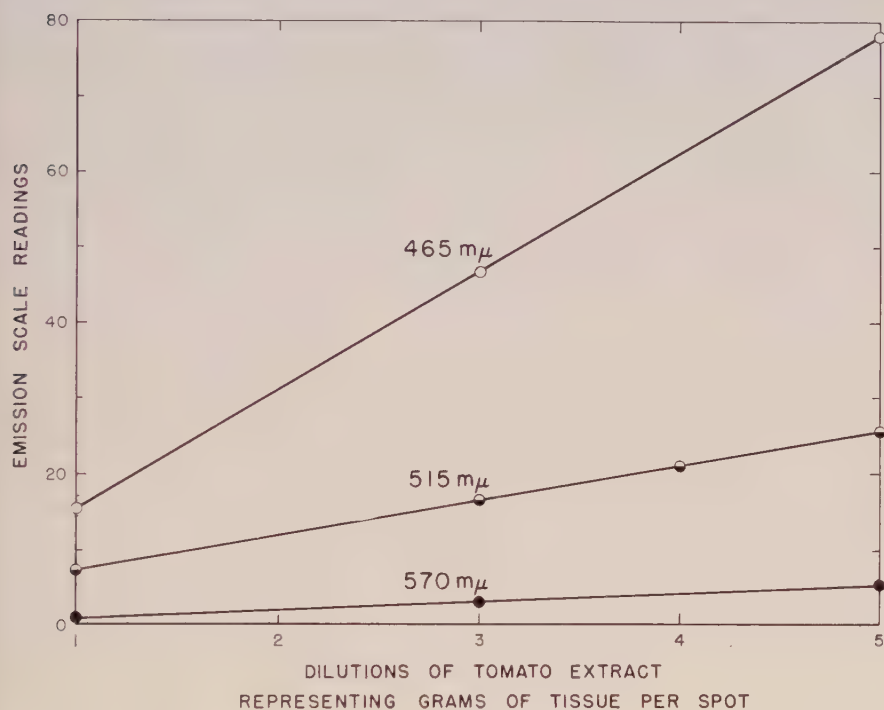


FIGURE 3. Emission of an unknown fluorescent substance extracted from tomato tissue and located at R_f 0.25 to 0.29 by means of an ultraviolet lamp. The photomultiplier was set at $\times 10$ for the 515 $m\mu$ and 465 $m\mu$ filters, and at $\times 1$ for the 570 $m\mu$ filter.

chromatographs are shown in Table II and Figure 3. These data show that a linear relationship exists between the fluorescence of the unknown substance and the amount of tissue represented by each chromatograph spot for tissue dilutions of 1 to 5. However, this is a much smaller range of dilution than that shown for IAA (0.5 to 400) in Figure 2.

The fluorescence measuring system just described makes possible the rapid detection and estimation of a fluorescent substance without the use of a developer (color reagent). Consequently, the substance may be eluted

from the paper in unchanged form after measuring its fluorescent emission. Measurements can also be made of fluorescent substances for which no satisfactory color reagent has been found.

A more elaborate setup was devised by Brown and Marsh (1) for the automatic measurement of light absorption and fluorescence on paper chromatograms. It consists of a Beckman Model DU spectrophotometer, a spacer, a scanning chamber, a strip-transporting mechanism, a photo-multiplier tube detector with amplifier and a strip chart recorder.

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IMPROVED APPARATUS AND PROCEDURES FOR SAMPLING AND ANALYZING AIR FOR FLUORIDES

R. MAVRODINEANU AND R. R. COE

SUMMARY

Descriptions are given of improved apparatus and procedures for sampling and analyzing air containing hydrofluoric acid (HF) and silicon tetrafluoride (SiF_4) gases in concentrations of less than one to several hundred parts per billion (10^9) of air. A closed type conical-shaped absorber of 500-ml. capacity containing 50 ml. of absorbing solution proved efficient [95 per cent recovery of the fluorine (F)] for absorbing fluorides from air passed through the absorber at rates up to 70 cu. ft. per hr. A smaller absorber (300 ml.) of similar design containing 40 ml. of absorbing solution was equally efficient with air speeds up to 40 cu. ft. per hr. and, when converted into a distilling unit without transferring the sample, gave 95 per cent recovery of F and an average blank of 1 μg . F. Both absorbers are easy to wash and are less subject to contamination than conventional types previously used.

A description is also given of an automatic time-clock sampling unit together with details of the back titration procedure used in fluorine analysis. Recent results indicate that direct titration of the sample with thorium nitrate and determination of the end point by means of a specially designed filter photometer constitute a much faster, more sensitive, and more precise method than visual titration with sodium fluoride (NaF).

INTRODUCTION

A study of the effects of air-borne fluorides on plants requires efficient apparatus and procedures for sampling and analyzing the air. This report describes equipment and procedures designed especially for sampling and analyzing air containing HF and SiF_4 gases. These two gases were obtained by atomization of solutions of HF and fluosilicic acid (H_2SiF_6) respectively, followed by passage through a thermal reactor under conditions previously described (3). Both gases (HF and SiF_4) are expressed as HF in the present report.

ABSORBER

After testing several kinds of absorbers, a closed type conical-shaped Pyrex glass container of 500-ml. capacity was devised (Fig. 1 A). Solution is added to the absorber by applying a slight vacuum on the left tube and is emptied by applying a slight pressure to the same tube. The absorber is designed to furnish a large surface contact of the incoming air with the solution, and consequently results in efficient absorption of the fluoride gases. The absorbing liquid is fluorine-free water obtained by passing distilled water through a demineralizing column. The volume of absorbing solution used (40 to 70 ml.) depends upon the length of time, temperature, relative humidity, and speed of air sampling.

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TABLE I

RECOVERY OF KNOWN AMOUNTS OF FLUORINE ADDED AS NaF, ACCORDING TO THE RATE OF FLOW AND TOTAL VOLUME OF AIR

| Rate of air flow, cu. ft. per hr. | Total volume of air, cu. ft. | Recovery of added fluorine, 40 μ g F in 50 ml. | |
|-----------------------------------|------------------------------|--|------------|
| | | μ g. F | Percentage |
| 20 | 20 | 37 | 93 |
| 40 | 40 | 39 | 98 |
| 60 | 60 | 40 | 100 |
| 80 | 80 | 37 | 93 |
| 80 | 240 | 36 | 91 |

The air to be analyzed is drawn at a predetermined metered speed through the absorber (Fig. 1 A) by means of a vacuum pump, entering through the tube on the right side and after bubbling through the absorbing solution passing out through the tube on the left side. Washing of the absorber is accomplished by connecting the left tube with a water vacuum pump and the right tube with a flask of boiling F-free water.

The efficiency of the absorber was determined by passing F-free air at different speeds through 50 ml. of absorbing solution containing known amounts of F as NaF (Table I), and by passing air containing F as SiF₄ at different speeds through absorbers containing 50 ml. of F-free water at the start (Table II). Data in Table I show that 91 to 100 per cent of the added F was recovered after passing 20 to 240 cu. ft. of air through the absorbing solution at speeds of 20 to 80 cu. ft. per hr. Data in Table II show that 90 to 98 p.p.b. of the fluorine was absorbed from 10 to 47 cu. ft. of air passed through the absorber at speeds of 10 to 47 cu. ft. per hr. Incomplete absorption resulted from the passage of 84 cu. ft. of F-containing air through the absorber in one hour. These and other tests indicate that good recovery of the fluorine present as HF or SiF₄ is obtained at rates of air flow up to about 70 cu. ft. per hr.

TABLE II

RECOVERY OF FLUORINE IN THE AIR (ADDED AS SiF₄*) ACCORDING TO RATE OF AIR FLOW THROUGH THE ABSORBER

| Rate of air flow, cu. ft. per hr. | Total volume of air, cu. ft. | Absorption of fluorine calculated as p.p.b. HF |
|-----------------------------------|------------------------------|--|
| 10 | 10 | 98 |
| 20 | 20 | 94 |
| 26 | 26 | 90 |
| 47 | 47 | 93 |
| 84 | 84 | 79 |

* An atomized solution of H₂SiF₆ was converted to SiF₄ gas by passage through a thermal reactor under conditions previously described (3).



FIGURE 1. Absorbers and absorbing-distilling unit. A. Closed type conical-shaped absorber of 500-ml. capacity made of Pyrex glass and containing glass beads to increase efficiency of absorption. B. Absorber of 300-ml. capacity having three outlets but otherwise similar to the absorber in A. C. Absorbing-distilling unit consisting of absorber (same as in B) set in an electric heating jacket controlled by a powerstat below at right, a condenser connected from above through a ground glass joint, and a Pyrex steam generator resting on a hot plate below. The thermometer registers a maximum of 150°C .

ABSORBING-DISTILLING UNIT

When interfering ions are known not to be present, the absorbed fluorine is titrated directly without being distilled, as described in another section of this report. However, when sulfur dioxide or other interfering substances are known to be present, the absorbed F is steam distilled from

a perchloric acid solution. For this purpose the apparatus¹ shown in Figure 1 B and C was designed so that the absorption and steam distillation of F could be performed in the same container and thereby eliminate possible losses due to transfer of all or part of the sample.

The absorber shown in Figure 1 B and C is slightly smaller (300 ml.) and has a different number of outlets, but is otherwise similar in design to the absorber shown in Figure 1 A. The air enters through the left tube, bubbles through the solution and glass beads and passes through the center outlet which is provided with a ground glass joint. The tube on the right is closed with a rubber stopper during sampling. After collection of the air sample, 40 ml. of 70 per cent perchloric acid are added to the absorber (Fig. 1 B) which is then converted into a distilling unit by connecting to a condenser above and to a steam generator below as shown in Figure 1 C. The distillation follows the same procedure previously described (4) with the exception that only 100 ml. of distillate are collected in a polyethylene beaker of 150-ml. capacity. The average recovery of F was 95 per cent and the average blank determination was 1 μ g. F.

AUTOMATIC SAMPLING UNIT

The automatic air sampling unit shown in Figure 2 was designed to give flexibility in sampling during each 24 hours of fumigation. Four samples can be taken simultaneously at the same or different rates, or samples can be taken at four predetermined periods during each 24 hours of fumigation. The unit shown in Figure 2 consists of a vacuum source, an electric time clock, a mercury safety valve, and a solenoid valve connected to an air meter.

Valves A and B are used for coarse and fine adjustment of the vacuum supply controlled by the flowmeter, D. The desired sampling time is pre-set on the time clocks, C₁, C₂, C₃, and C₄, which electrically operate the solenoid valves, E₁, E₂, E₃, and E₄. The T-form mercury filled safety valves, G₁, G₂, G₃, and G₄, are used to release any pressure which may develop in the absorber-meter closed system during nonsampling periods. Four dry meters, H₁, H₂, H₃, and H₄, record the volumes of air passing through the absorbers.

TITRATION PROCEDURE

Reagents required:

Fluorine-free distilled water—adjusted to pH 3.0 ± 0.05 with HCl.
Indicator 1 per cent sodium alizarinsulfonate (stock) diluted to 0.01 per cent (freshly prepared every three days).

¹ Glass equipment shown in Figure 1 was made according to specifications by the Yonkers Laboratory Supply Company, Yonkers, N. Y.

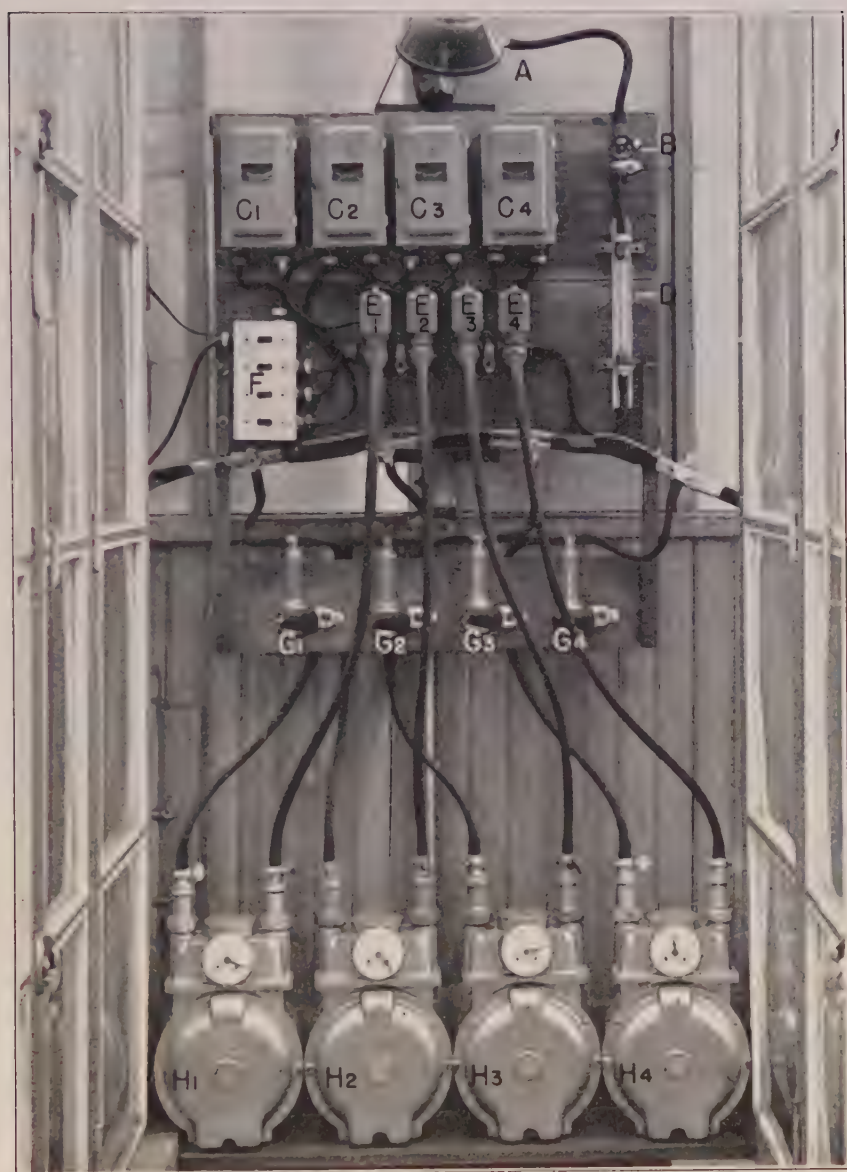


FIGURE 2. Automatic sampling unit. A and B: valves for coarse and fine adjustment of the vacuum; C₁, C₂, C₃, and C₄: electric time clocks (Tork Clock Co., Inc., Mount Vernon, N. Y.); D: flowmeter (tube size 6-15-2, Ace Glass Co., Vineland, N. J.); E₁, E₂, E₃, and E₄: solenoid valves (Asco, Catalog No. 82102, Orange, N. J.); F: four switches controlling the time clocks; G₁, G₂, G₃, and G₄: T-form safety valves filled with mercury (Pyrex glass); H₁, H₂, H₃, and H₄: dry meters for measuring volume of air sample (Sprague #1A, Bridgeport, Conn.).

Thorium nitrate (stock)—2.00 g. $\text{Th}(\text{NO}_3)_4 \cdot 4\text{H}_2\text{O}$ per liter of distilled water.

Thorium nitrate titrating solution—0.20 g. $\text{Th}(\text{NO}_3)_4 \cdot 4\text{H}_2\text{O}$ per liter of distilled water.

Sodium fluoride (stock)—2.210 g. Baker's C.P. NaF (dried at $105^\circ\text{C}.$) per liter of distilled water. The stock solution containing 1 mg. F per ml. is diluted to 10 $\mu\text{g}.$ F per ml. for use in titrating.

All solutions were kept in the refrigerator when not in use. Stock solutions were frequently checked for possible change in concentration.

The entire sample or a suitable aliquot is analyzed (1, 2, 6), depending upon the quantity of F absorbed. With small quantities of F, all of the solution is transferred from the absorber to a 150-ml. polyethylene beaker and the absorbing flask washed once with F-free water and made up to 100 ml. A recovery of 90 per cent was obtained with no washing and 98 per cent with one washing of 25 ml.

Samples with larger amounts of F are transferred to a 250-ml. polyethylene bottle and made up to a known volume. An aliquot is pipetted into a polyethylene beaker of 150-ml. capacity and adjusted to pH 3.0 ± 0.05 with 0.1 *N* HCl and 0.1 *N* NaOH using a Beckman pH meter.

After matched 100-ml. tall form Nessler tubes are rinsed with distilled water adjusted to pH 3.0, the test solutions are transferred from the beakers to the Nessler tubes, and 2 ml. of 0.01 per cent sodium alizarinsulfonate are added to each tube. The volume is then made up to the 100-ml. mark with distilled water of pH 3.0.

Thorium nitrate is added to the first unknown until a shade of pink color is reached which a given analyst selects as most suitable for matching. Mixing is accomplished by several consecutive inversions of capped Nessler tubes. After the desired pink color is obtained in the first Nessler tube, an equal amount of thorium nitrate is added to the blank (pH 3.0).

The last step is the addition of small known amounts of NaF solution to the blank until the color of the blank is the same as that of the unknown. When there are several unknowns, the darker one is adjusted first. In case of higher fluoride content (over 10 $\mu\text{g}.$ F in the Nessler tube), it is advisable to add to the blank an amount of the standard NaF solution equivalent to about 80 per cent of the fluoride expected to be present in the sample aliquot titrated, before the addition of the thorium nitrate.

The concentration of HF in the air sample is calculated as follows (conditions of $25^\circ\text{C}.$ and 760 mm. Hg were chosen arbitrarily for convenience of calculation):

$$\frac{\text{Ml. NaF} \times \text{aliquot factor} \times 45^2}{\text{Cu. ft. air}} = \text{p.p.b. HF by volume}$$

² Given to the nearest whole number.

This short formula is derived from:

$$\frac{\text{Aliquot factor} \times \text{g. F} \times \frac{\text{mol. wt. HF}}{\text{at. wt. F}} \times \frac{1}{\text{mol. wt. HF}} \times 22.4 \times \frac{1}{28.32} \times 10^9}{\text{Cu. ft. air absorbed (corrected to } 0^\circ \text{ C. and 760 mm. Hg)}} \\ = \text{p.p.b. HF at } 0^\circ \text{ C. and 760 mm. Hg}$$

in which mol. wt. HF = 20.0, at. wt. H = 1.0, mol. vol. equivalency factor (liters) = 22.4, and conversion factor (cu. ft. to liters) = 28.32. It is assumed that there is little association of HF at the great dilutions measured here.

TABLE III
APPROXIMATE MINIMUM TITRATION FOR PREPARED SOLUTIONS OF NaF REQUIRED
TO OBTAIN A RECOVERY OF FLUORINE NOT EXCEEDING AN ERROR OF
± 10 PER CENT

| Fluorine added as NaF, μg. F | Calculated titration, ml. NaF | Recovery of fluorine | | |
|------------------------------------|-------------------------------------|-----------------------|-----------------------|-----------------------------------|
| | | Titration, ml. NaF | Equivalent, μg. F. | Per cent, recovery, average |
| 0.6 | 0.06 | 0.10 0.11 | 1.0 1.1 | 175 |
| 1.3 | 0.13 | 0.20 0.17 | 2.0 1.7 | 142 |
| 4.0 | 0.40 | 0.34 0.39 | 3.4 3.9 | 91 |
| 6.0 | 0.60 | 0.65 0.68 | 6.5 6.8 | 110 |
| 11.0 | 1.10 | 1.00 1.00 | 10.0 10.0 | 91 |

In order to determine the minimum amount of F necessary for obtaining analytical results with an error of 10 per cent or less, titrations were performed by two operators on different known quantities of F. Results in Table III show that a minimum volume of 0.4 ml. NaF titrating solution corresponding to 4 μg. F is required to give recoveries within 10 per cent of the known quantities of F used.

If the approximate concentration in F is known, it is easy to calculate the amount of air that should be sampled by using the short formula shown above. Generally, more than the calculated minimum volume of air is drawn through the absorber, and consequently most titrations require more than 0.4 ml. NaF. However, the volume of air sample containing relatively low concentrations of HF (e.g., 3 p.p.b. or less) should not be

greatly in excess of that needed to give measurable quantities of fluoride by the titration method.

An improved method of titration now being used makes possible the rapid determination of F in air samples and in distillates of tissue samples with much greater precision than by the procedures just described for back titrating with NaF. The method involves direct titration with thorium nitrate and the determination of the end point by means of a specially designed filter photometer. Experimental values fall on a straight line from 1 μ g. F in 250 ml. upward, showing that the proportionality between the F content of the sample and the volume of thorium nitrate used holds even for the smallest measurable volume of thorium nitrate delivered from a microburette. A description of the filter photometer and the results obtained with its use in fluorine analysis appear in another report (5).

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PHOTOELECTRIC END-POINT DETERMINATION IN THE TITRATION OF FLUORIDES WITH THORIUM NITRATE

R. MAVRODINEANU AND J. GWIRTSMAN

SUMMARY

A description is given of a photoelectric filter photometer functioning as a comparator for the determination of the end point in the titration of fluorides (F) with thorium nitrate $[\text{Th}(\text{NO}_3)_4]$ in the presence of sodium alizarinsulfonate. It is a balance type instrument consisting of a nearly monochromatic light source, two glass cells for the solutions, and two barrier-layer photoelectric cells connected in opposition. A mirror galvanometer mounted in parallel acts as a zero indicator. The instrument is directly supplied by the 115-volt line and is stable and sensitive. Its use makes possible the titration of $1\text{ }\mu\text{g. F}$ in 250 ml. of solution, one determination requiring 10 minutes. When equipped with adequate supplementary parts it can also be used for the transmission measurements of colored solutions as well as a nephelometer or fluorometer.

INTRODUCTION

The procedure used in this laboratory for the determination of fluorides in plant tissue and in air is based on the Willard and Winter method (8). In this method the fluoride is isolated in the form of fluosilicic acid after a perchloric acid steam distillation at 135°C . (6). The determination of the fluoride ion itself is carried out by means of a titration with $\text{Th}(\text{NO}_3)_4$ in the presence of sodium alizarinsulfonate at pH 3.0. This operation may be performed by a direct or back titration. In the direct titration an aliquot of the distillate adjusted to pH 3.0 is pipetted into a 250-ml. cylinder and, after addition of 2 ml. of sodium alizarinsulfonate solution, is titrated directly with a standard solution of $\text{Th}(\text{NO}_3)_4$ to a faint pink, matched with the color obtained in a second cylinder of the same size by adding distilled water adjusted to pH 3.0, 2 ml. of indicator, and a few drops of $\text{Th}(\text{NO}_3)_4$ solution. The back titration procedure is described elsewhere (4).

In both procedures visual comparison of colors is time-consuming, is fatiguing to the operator, and involves subjective errors. An instrumental measurement of the end point is preferable and has been used in other laboratories (7). For example, photometric fluoride titrators have been used for three years at the Aluminum Research Laboratories, Aluminum Company of America, New Kensington, Pa.¹ In order to improve the method of titrating fluorides in our laboratory, a similar photoelectric comparator was designed especially for this purpose. The present report describes this instrument and its use for determining the end point in the titration of F and suggests uses for the transmission measurements of colored solutions and for measuring turbidity and fluorescence.

¹ Personal communication from Dr. M. L. Moss, Assistant Chief, Analytical Chemistry Division.

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APPARATUS

Commercially available photometers were not suitable for the contemplated method of titrating fluorides. Consequently, an apparatus was constructed similar to one used previously (5), but with the incorporation of several new elements, including a Universal instrument bench supplied by Meyer Opticraft, Inc., New York, N. Y. The instrument shown in Figure 1 is a balance type photometer and includes a light source mounted in an enclosure placed in the middle of the optical bench. Two lenses are sym-

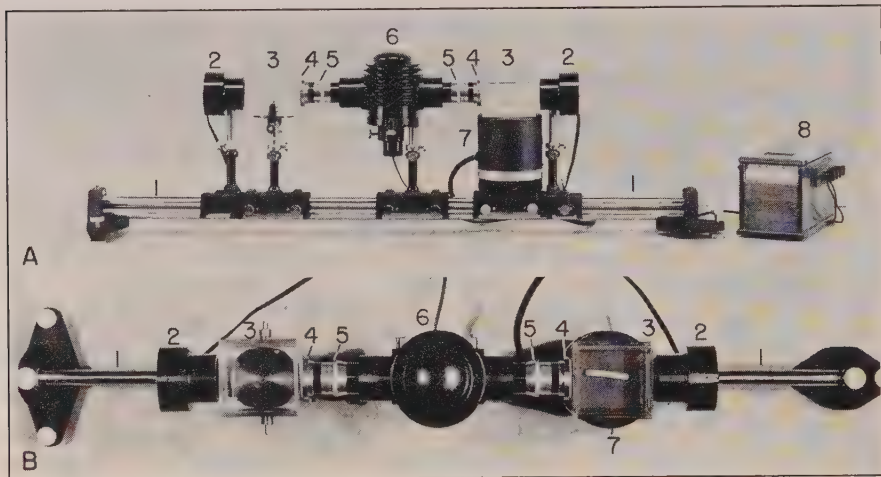


FIGURE 1. Photoelectric filter photometer. A. Side view; B. top view. A and B: 1. Optical bench; 2. Weston Photronic photocells, Model 594; 3. cells for solution with plane parallel glass plates 250-ml. capacity and 10 cm. long; 4. green colored gelatin filters having a maximum transmission at $520\text{ m}\mu$; 5. Meyer Trioplan lenses, 3 in., $F: 2.8$, provided with adjustable iris diaphragm; 6. lamp housing containing a 6-volt and 4-amp. bulb supplied through a transformer directly from the 115-volt line; 7. magnetic stirrer (Arthur H. Thomas Co., Philadelphia, Cat. No. 9235-R); 8. Leeds and Northrup mirror galvanometer with internal reflection—sensitivity = 50 divisions per microampere, resistance = 1120 ohms, period = 3.1 sec. The described parts are placed on the optical bench on convenient riders, adjustable in position and height; the vertical columns are provided with locking collars.

metrically located on either side of the enclosure. They are provided with adjustable iris diaphragms and two colored gelatin filters having a maximum transmission at a wave length of $520\text{ m}\mu$ (1, 3). The slightly convergent light beams (2, p. 118) pass through the glass cells having a capacity of 250 ml. and a length of 10 cm. The left cell is supported by a stand; the right one is placed on a magnetic stirrer mounted on the optical bench. Two barrier-layer photoelectric cells, mounted in suitable enclosures, are placed at the two ends of the optical bench and receive the light beams which pass through the solutions. The photocells are connected in opposition (2, p. 99), a mirror galvanometer being mounted in parallel. The latter

is shunted by Ayrton shunt (Fisher Scientific Company Catalog No. 11-506-15) which permits stepwise variation of the sensitivity over a range of 1 to 500 (not shown in the figure).

When not in use the galvanometer is bypassed by means of a switch. In order to operate the instrument at full light, the glass cells containing the solutions are placed in aluminum enclosures (not shown), the interior walls of which are coated with a flat black paint.

TITRATION PROCEDURE

Reagent:

Fluorine-free water obtained by passing distilled water through a demineralizer.

For the titration of F in the range 0 to 100 $\mu\text{g.}$:

$\text{Th}(\text{NO}_3)_4$ solution containing 0.20 g. tetrahydrate per liter or 0.00145 *N*.

Sodium fluoride (NaF) stock solution containing 1 mg. F per ml.

Sodium fluoride standard solution containing 10 $\mu\text{g.}$ F per ml.

Sodium alizarinsulfonate indicator 0.050 g. per 500 ml. of water (0.01 per cent).

Hydrochloric acid 0.1 *N* and 0.01 *N* and sodium hydroxide 0.1 *N* and 0.01 *N* for pH adjustment.

For the titration of F in the range 0 to 1000 $\mu\text{g.}$:

$\text{Th}(\text{NO}_3)_4$ 0.01 *N* solution obtained from a standard 0.1 *N* $\text{Th}(\text{NO}_3)_4$ solution standardized against a known NaF solution.

Sodium fluoride solution containing 100 $\mu\text{g.}$ F per ml.

Sodium alizarinsulfonate indicator, 0.175 g. per 500 ml. of water (0.035 per cent).

Hydrochloric acid 0.1 *N* and 0.01 *N* and sodium hydroxide 0.1 *N* and 0.01 *N* for pH adjustment.

For a direct titration the blank is prepared by adding to the left cell (Fig. 1) 250 ml. of fluorine-free water adjusted to $\text{pH } 3.0 \pm 0.05$ and 2 ml. of indicator. An equal volume (250 ml.) of fluoride-containing test solution (distillate) in the right cell is adjusted to $\text{pH } 3.0 \pm 0.05$ and then 2 ml. of indicator are added. At this step the solutions in both cells are green. The light source is turned on, the galvanometer connected, and the optical system balanced by means of the iris diaphragms until the galvanometer reads zero. The Ayrton shunt is then switched to maximum sensitivity ($\times 1$) for titrations ranging from 0 to 100 $\mu\text{g.}$ F and to position $\times 10$ (sensitivity reduced by a factor of 10) for titrations ranging from 0 to 1000 $\mu\text{g.}$ F. The F-containing solution is now titrated dropwise with a standard $\text{Th}(\text{NO}_3)_4$ solution using a microburette, and the reaction is completed when the galvanometer spot is deflected from its zero position to a scale reading of 25. In the absence of F, 0.11 ml. of the standard 0.00145 *N*

$\text{Th}(\text{NO}_3)_4$ solution is required to produce this deflection, and this volume is always deducted from the results obtained in the analyses.

The same optical system is used for back titration, but in this case the left cell containing the "blank" is placed on a second magnetic stirrer (not shown in the figure).

The two cells containing the solutions are filled and the optical system balanced as previously described. To the right cell standard $\text{Th}(\text{NO}_3)_4$ solution is added from the microburette until the galvanometer spot is displaced from 0 to 25. The same volume of $\text{Th}(\text{NO}_3)_4$ solution is then added to the left cell and, by means of another microburette, this solution is titrated with a standard sodium fluoride solution until the galvanometer spot is brought back to the zero position. During both direct and back titrations the magnetic stirrers are in continuous operation.

RESULTS AND DISCUSSION

The results obtained with direct titration of solutions containing known amounts of fluorine are shown in Figure 2. Figure 2, curve A, presents the

TABLE I
BACK TITRATION OF SOLUTIONS CONTAINING KNOWN AMOUNTS OF FLUORIDE

| 0 to 100 $\mu\text{g. F}$ | | | 0 to 1000 $\mu\text{g. F}$ | | |
|--|--|--|--|--|--|
| $\mu\text{g. F}$ present in solution | Ml. $\text{Th}(\text{NO}_3)_4$ 0.00145 <i>N</i> used | $\mu\text{g. F}$ found in back titration with NaF | $\mu\text{g. F}$ present in solution | Ml. $\text{Th}(\text{NO}_3)_4$ 0.01 <i>N</i> used | $\mu\text{g. F}$ found in back titration with NaF |
| — | 0.11 | — | — | 0.04 | — |
| 10 | 0.54 | 10 | 10 | 0.10 | 10 |
| 30 | 1.52 | 30 | 100 | 0.65 | 100 |
| 50 | 2.57 | 50.7 | 300 | 1.80 | 297 |
| 70 | 3.50 | 71.5 | 500 | 2.84 | 491 |
| 90 | 4.61 | 91.2 | 700 | 3.70 | 690 |
| 100 | 5.23 | 97.5 | 900 | 4.46 | 883 |
| | | | 1000 | 4.67 | 970 |

results obtained in the titration of known amounts ranging from 0 to 100 $\mu\text{g. F}$. These values, after deduction of 0.11 ml., are represented graphically by a straight line which intercepts at the origin.

Similar results are presented in Figure 2, curve B, for known amounts ranging from 0 to 1000 $\mu\text{g. F}$. In this case the values obtained after deduction of 0.04 ml. are represented graphically by a straight line up to about 300 $\mu\text{g. F}$ which at higher concentrations curves towards the abscissa.

The results obtained with back titration of solutions containing known amounts of fluorine are presented in Table I.

The filter photometer described above showed remarkable stability.

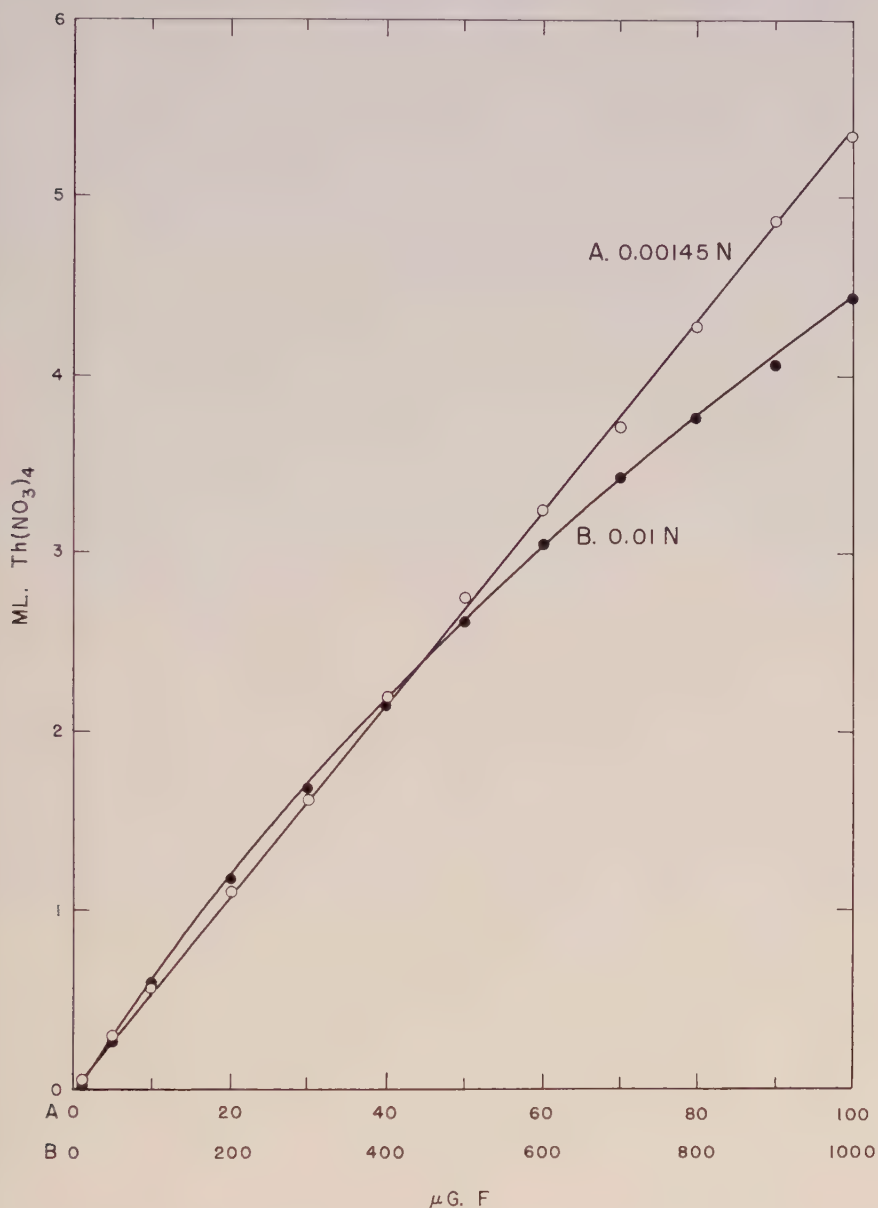


FIGURE 2. Calibration curves for the titration of fluorides with $\text{Th}(\text{NO}_3)_4$. A and B. Linearity shown between the concentration of $\text{Th}(\text{NO}_3)_4$ and F for amounts of F up to about 300 μg , and a falling off with higher amounts.

After balancing the optical system the galvanometer spot did not vary more than ± 2 divisions, even though the light source was directly supplied from the 115-volt line without the use of a voltage stabilizer.

The sensitivity of the instrument was tested by starting from a balanced system after fluorine-free water adjusted to pH 3.0 and 2 ml. of indicator were added to each cell. One drop of the 0.00145 *N* Th(NO₃)₄ solution added to the right cell displaced the spot of the galvanometer, switched to its maximum sensitivity, for 5 to 6 divisions. Using a drop of the 0.01 *N* Th(NO₃)₄ solution, the spot of the galvanometer with its sensitivity reduced ten times was displaced by 15 divisions.

Comparison between visual back titration and photoelectric direct titration showed that the latter is at least three times faster. Furthermore, the use of the photoelectric comparator eliminates subjective errors and does not involve visual fatigue of the operator.

Besides its use for fluoride analyses, this photoelectric filter photometer may be used to measure the end point of any titration involving color changes. When equipped with adequate supplementary parts, transmission measurements can be made of colored solutions in the visible range for a wide range of light paths. Turbidity and fluorescence measurements can also be performed. Such applications will be the subject of a future paper.

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COMPARISON OF ETHYLENEDIOXYPHENYL AND METHYLENEDIOXYPHENYL COMPOUNDS AS EXTENDERS FOR PYRETHRINS

EDWARD A. PRILL AND WILLIAM RICHARD SMITH

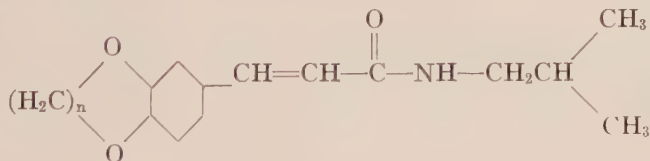
SUMMARY

Numerous methylenedioxyphenyl compounds have been previously found to be effective as synergists or extenders for pyrethrins. It was deemed of importance to determine whether ethylenedioxyphenyl compounds may also be active as extenders for pyrethrins. *N*-Isobutyl-3,4-methylenedioxybenzamide (fagaramide) and 2-(3,4-methylenedioxyphenyl)-5,5-diethyl-*m*-dioxane were prepared as active reference compounds of the methylenedioxyphenyl type. The respectively corresponding ethylenedioxyphenyl compounds which were prepared and tested were *N*-isobutyl-1,4-benzodioxan-6-acrylamide and 2-(1,4-benzodioxan-6-yl)-5,5-diethyl-*m*-dioxane. The ethylenedioxyphenyl compounds were found to be inactive as extenders when tested against houseflies (*Musca domestica* L.).

INTRODUCTION

During the last fifteen years numerous compounds having the common feature of containing the methylenedioxyphenyl nucleus in their structures have been found to be effective synergists or extenders for pyrethrins (13). Still it is not known what specific characteristic is possessed by the methylenedioxyphenyl nucleus which can make possible this particular biological activity of compounds containing this nucleus in combination with a wide but not unlimited variety of organic radicals. With the objective of throwing light on this problem, compounds containing related nuclei are being studied in this laboratory.

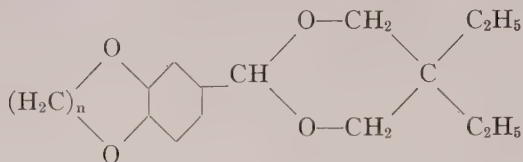
The present paper presents the syntheses and the results of comparative tests of two compounds containing the methylenedioxyphenyl nucleus (I and III) and two corresponding compounds containing the ethylenedioxyphenyl, or more properly designated, 1,4-benzodioxan nucleus (II and IV).



I $n = 1$, *N*-Isobutyl-3,4-methylenedioxybenzamide (fagaramide)

II $n = 2$, *N*-Isobutyl-1,4-benzodioxan-6-acrylamide

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III $n=1$, 2-(3,4-Methylenedioxyphenyl)-5,5-diethyl-*m*-dioxane

IV $n=2$, 2-(1,4-Benzodioxan-6-yl)-5,5-diethyl-*m*-dioxane

The compound represented by I, fagaramide, has been known for a long time (2) and has been reported (6) to be active as a synergist for pyrethrins. The compound represented by III is a cyclic acetal of piperonal and might be expected to be similar in activity to some related cyclic acetals of piperonal which were previously found (10) active as synergists. Although these methylenedioxyphenyl compounds may not be as active as some of the best synergists or extenders now available, they are sufficiently active for use in comparative tests, and furthermore, their ethylene-dioxyphenyl counterparts can be synthesized without difficulty.

PREPARATION OF COMPOUNDS

N-Isobutyl-3,4-methylenedioxy-cinnamamide (fagaramide) (I). 3,4-Methylenedioxy-cinnamic acid was made by the Doebner modification of the Knoevenagel condensation as outlined by Johnson (8, p. 249) with the difference that dry morpholine was used instead of piperidine as the catalyst. The acid was converted into its acid chloride by refluxing with thionyl chloride in benzene solution. After distilling off the excess thionyl chloride and benzene under a water pump vacuum the 3,4-methylenedioxy-cinnamoyl chloride was caused to react with an excess of isobutylamine in benzene solution by allowing it to stand in a warm place overnight. The solution was washed successively with dilute HCl, dilute KOH and water. The amide was isolated and after recrystallization from ethyl acetate had a m.p. of 119 to 120°. The literature (2, p. 299) reports a m.p. of 119.5°.

1,4-Benzodioxan. This was prepared according to Ghosh's (7, p. 1591) procedure with the following modifications: copper powder was used as the catalyst, the reaction mixture was diluted with anisole and the reaction run at reflux temperature (115–125°) for 20 hours with occasional stirring to prevent caking. The purified product distilled at 212–215°.

1,4-Benzodioxan-6-aldehyde. A mixture of 16.4 g. (.12 mole) of 1,4-benzodioxan, 23 g. (.17 mole) of *N*-methylformanilide, and 26.2 g. (.17 mole) of POCl₃ was heated on a steam bath for 2 hours. The reaction mixture was then poured into crushed ice, made alkaline with Na₂CO₃ and extracted with ether. The aldehyde was extracted from the ether solution by means of saturated aqueous NaHSO₃ solution, then precipitated by addition of Na₂CO₃ solution. Recrystallization from ethyl ether-petroleum ether mixture gave a 37 per cent yield of very light yellow needle-like crys-

tals having a m.p. of 51–51.5°. The reported (5, p. 373) m.p. is 51.5°.

1,4-Benzodioxan-6-acrylic acid. This acid was made similarly to the above mentioned 3,4-methylenedioxybenzoic acid (8). A mixture of 9 g. (.05 mole) of 1,4-benzodioxan-6-aldehyde, 10.2 g. (.1 mole) of malonic acid, 30 ml. of dry pyridine and 1 ml. of dry morpholine was heated on a steam bath for 3 hours. The product was isolated in a good yield and after recrystallization from dilute ethanol had a m.p. of 190–191°. *Neutral equiv.* Calcd. for $C_{11}H_{10}O_4$: 206.2. Found: 200.

Dobrowsky (4, p. 134) using a drop of conc. H_2SO_4 as the condensing agent obtained this acid as a substance melting at 182°. Following his method a product melting at 182° was obtained the first time, but a later batch which had been repeatedly recrystallized from dilute ethanol had a m.p. of 190–191° and showed no depression of the m.p. when mixed with the acid described in the previous paragraph.

N-Isobutyl-1,4-benzodioxan-6-acrylamide (II). This amide was made similarly to the above described *N*-isobutyl-3,4-methylenedioxybenzamide using the acid chloride made from 12.7 g. of the 190–191° melting 1,4-benzodioxan-6-acrylic acid. The amide partially precipitated out of the benzene solution during the process of washing with dilute HCl and KOH solutions. After recrystallization from dilute ethanol the amide had a m.p. of 144–144.5°. *Anal.* Calcd. for $C_{15}H_{19}O_3N$: 5.34% N. Found: 5.23% N.

Cyclic acetals. The following two cyclic acetals were prepared by the previously described method (10) involving the azeotropic removal of water as it is formed in the condensation of an aldehyde with a glycol in refluxing benzene solution. For this method a Dean and Stark type moisture trap is placed between the flask and the reflux condenser.

2,2-Diethyl-1,3-propanediol was obtained from Carbide and Carbon Chemicals Company and was purified by recrystallization from toluene to give a product having the reported (12) m.p. of 61–61.5°. This purified glycol was used in the syntheses of the following cyclic acetals.

The molecular refractions of the cyclic acetals were determined in the usual manner (11, pp. 40–45) and serve to identify the compounds.

2-(3,4-Methylenedioxyphenyl)-5,5-diethyl-m-dioxane (III). This compound was prepared from 30 g. (.2 mole) piperonal, 39.6 g. (.3 mole) of 2,2-diethyl-1,3-propanediol, and 1 g. of *p*-toluenesulfonic acid in 200 ml. of benzene. The product obtained in 80 per cent yield had the following characteristics: b.p. 155–156° at 3 mm., n_D^{25} 1.5238, d_{25} 1.1465. *Mol. refraction.* Calcd. 70.05. Found: 70.43.

2-(1,4-Benzodioxan-6-yl)-5,5-diethyl-m-dioxane (IV). This compound was prepared from 12 g. (.073 mole) of 1,4-benzodioxan-6-aldehyde, 18.4 g. (.14 mole) of 2,2-diethyl-1,3-propanediol, and 1.5 g. of *p*-toluenesulfonic acid in 200 ml. of benzene. The product was found to have the following

characteristics: b.p. 173–180° at 3 mm., n_D^{25} 1.5311, d_{25}^4 1.1359. *Mol. refraction*. Calcd. 74.67. Found: 75.73.

TESTS AND RESULTS

The compounds with added pyrethrins were tested against houseflies (*Musca domestica* L.) by the large group Peet-Grady method (9). The composition of the test solutions and the results of the tests are shown in Table I. The solvent for the test solutions of group A consisted of 80 parts Deo-base (a petroleum distillate), 15 parts acetone, and 5 parts xylene by volume. It was not feasible to test the amides in group A at concentrations greater than 0.2 g. per 100 ml. because of their limited solubilities. Careful observations were made to assure that no compound precipitated out of the solutions before they were tested. The solvent for the test solutions of group B was pure Deo-base.

TABLE I
COMPARATIVE ACTIVITY AS EXTENDERS FOR PYRETHRINS OF METHYLENEDIOXYPHENYL (I AND III) AND ETHYLENEDIOXYPHENYL (II AND IV) COMPOUNDS AS SHOWN BY PEET-GRADY TESTS

| Group | Compound* | Test solution, 0.1 g./100 ml. pyrethrins plus com- pound, g./100 ml. | 10 Min. knockdown, % | 24 Hr. kill, % |
|-------|-----------|---|-------------------------|-------------------|
| A | I | 0.2 | 92 | 66 |
| | II | 0.2 | 91 | 38 |
| | — | None | 91 | 32 |
| B | III | 1.0 | 97 | 65 |
| | IV | 1.0 | 97 | 21 |
| | — | None | 95 | 19 |

* See text for compounds represented by the Roman numerals.

The results represent the averages of four replicate tests. The flies were rather resistant at the time these tests were made but this does not detract from the validity of the comparative results.

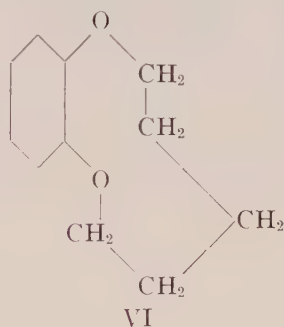
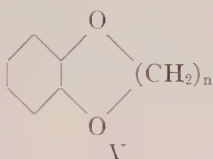
Fagaramide (I) plus pyrethrins gave a greatly increased kill over that of pyrethrins alone, therefore supporting the previous report (6) that it is a synergist. Its ethylenedioxyphenyl analogue (II), however, was essentially ineffective in admixture with pyrethrins.

The compound represented by III was found to be quite active similar to some other previously reported (10) related cyclic acetals of piperonal, whereas the ethylenedioxy analogue (IV), which is a cyclic acetal of 1,4-benzodioxan-6-aldehyde, was found to be inactive.

DISCUSSION

The foregoing experiments were prompted, in part, by a publication by Baddeley *et al.* (1) on a purely physico-chemical subject. These authors

have shown that the rates of nuclear bromination in a series of compounds represented by V were lower than the rate of bromination of veratrole (*o*-dimethoxybenzene) when n was 1, 2, or 3 but were higher when n was a larger number such as 5. A somewhat similar relationship was shown (1, 3) for the rates of exchange of nuclear hydrogen for deuterium in these compounds. These differences in the reaction rates were considered to be related to possible configurations around the ether groups (1). Thus when n is 5 the molecule can assume the configuration represented by VI in which the lower ether group is "facing" the other one, or in other words, the lower ether oxygen atom with its attached valence bonds is rotated through approximately 180° from the position it has in V when n is 1 or 2. Such a "facing" of ether groups would not be possible in the smaller rings but it could readily occur in compounds such as veratrole in which the ether oxygens are not contained in a ring structure.



This similarity of the methylenedioxy nucleus (V, $n = 1$) and the ethylenedioxy nucleus (V, $n = 2$) in regard to their reactivities in certain electrophilic substitution reactions and the presumably necessarily restricted configurations around the ether groups, suggested to the writers that possibly these nuclei might also have similar influences on biological activity. The results of the present tests, however, indicate that such is not the case as far as pyrethrin extenders are concerned. Therefore, in the case of the many methylenedioxyphenyl compounds which are active as synergists or extenders for pyrethrins, the activity is probably dependent upon some characteristic of the methylenedioxyphenyl nucleus other than those it has in common with the ethylenedioxyphenyl nucleus.

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MORPHOLOGICAL AND PHYSIOLOGICAL STUDIES OF DIPLOID AND TETRAPLOID *PLANTAGO OVATA* FORSK.

CLYDE CHANDLER AND LELA V. BARTON

SUMMARY

Colchicine-induced tetraploid plants of *Plantago ovata* which exhibit more desirable characteristics than the corresponding diploids for the commercial production of psyllium have a tendency to produce a dark brown exudate that reaches its maximum as the plants approach maturity. This is associated with a form of leaf injury. Up to 97 per cent of a tetraploid population may exude the material, but few diploids develop it. This phenomenon has prompted morphological and physiological comparisons of the diploid and tetraploid plants.

Morphological studies revealed that the tetraploids possess broader and thicker leaves composed of larger cells than the diploids. The former are also characterized by larger stomata and broader epidermal hairs. The exudate has been found in both lower and upper epidermal cells, as well as in all parenchymatous cells in the affected region.

It has been impossible to correlate the appearance of the exudate with mechanical or fumigant injuries; insect or mite feeding; or bacterial, fungus or virus infection.

Tetraploid leaves had a higher moisture content and a lower transpiration rate per gram of dry weight than those of the diploid. The osmotic pressure of the cell sap of the tetraploid was consistently lower than that of the diploid with a tendency for the difference to increase as the plants increased in age from 21 to 54 days. High humidity prevented formation of the exudate. Although the cause of exudate formation in the tetraploids has not been determined with certainty, it may be associated with the water relations of the plants.

Physiologic responses of diploids and tetraploids to other environmental conditions, while showing no direct relation to exudate formation, did indicate differences in growth and flowering. In general, development of tetraploid plants was delayed as compared with diploids. Details are given of reactions to a deficiency of potassium, phosphorus or nitrogen, or an excess of the latter in nutrient solution. Also growth and flowering was observed in the greenhouse in different soil types, with deficient, normal and excessive supplies of water and night temperatures of 5°, 10°, 21°, or 30° C. A long photoperiod (approximately 18 hours) hastened flowering of both diploid and tetraploid plants, but the first flower of the latter appeared a week later than those of the former. Under an eight-hour photoperiod, flowering of both forms was delayed for the same length of time.

The color of the exudate was found to be due to the presence in the cell sap of the glycoside, aucubin. This substance occurred normally in both diploid and tetraploid plants. The difference between the two forms in exudate formation does not appear to be due to the amount of aucubin, or to the type or amounts of amino acids or sugars in the cell sap, as determined by chromatographic methods.

INTRODUCTION

Many tetraploid plants of *Plantago ovata* Forsk., induced by soaking diploid seeds in aqueous solutions of colchicine, have been found to de-

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velop a dark brown exudate on the leaves (3). The appearance of this exudate (Fig. 1 B) accompanies an injury to the leaves, which may be killed as a result. The exudate may be produced on either the upper or the lower surface of the leaf and is frequently associated with curling (Fig. 1 A, C, D). Although up to 97 per cent of a tetraploid population may develop this exudate, very few diploids have been found to possess it. Such an exudate has not been reported in the literature. This paper describes morphological and physiological studies initiated to compare the characteristics of diploid and tetraploid plants and to determine possible differences which might account for the exudation phenomenon.

MORPHOLOGY

The tetraploid plants as previously reported (3) were more robust than diploids of the same age. Leaves were longer, wider and thicker, flowering scapes were thicker and taller, and flower spikes heavier in the tetraploids. All seedlings produced viable seed, though fertility was reduced 26 per cent in tetraploids. Tetraploid seed were 36.0 per cent heavier than diploids and produced 22.5 per cent more mucilage. These characteristics of the tetraploid were of decided commercial interest.

Morphological studies of the tetraploid plants show that not only are the leaves broader and thicker than the diploids but cells are larger (Fig. 2 A, B). Tetraploids may be distinguished easily from diploids by the increase in size of the stomata and also by broader epidermal hairs.

When the exudate is visible within the leaf but has not appeared externally as a drop on the surface, sections in the darkened area show an accumulation of the exudate material in the epidermal cells and in the first three rows of the parenchyma (Fig. 2 D). After a small drop of the exudate appears on the upper leaf surface, a small region of tissue is involved but no disintegration of tissue can be detected at this stage (Fig. 2 E). However, if the drop is of considerable size and appears on both the upper and the lower epidermis, a complete breakdown of tissue may occur (Fig. 2 C). This injury may be in the region of the vascular bundle but is not always associated with the vascular tissue. This may be due to some external injury rather than an internal destruction of tissue since leaves which exhibit the greatest quantity of exudate may not show any marked degree of disintegration (Fig. 2 F). In such a case both upper and lower epidermal cells as well as all parenchymatous cells in the region affected contain this brown substance which has been identified as a degradation product of aucubin. Frequently this exudate is found in spaces between the cells and may therefore be either inter- or intracellular.

PHYSIOLOGY

Observations have been made on a total of 675 diploids and 654 tetraploids grown in the greenhouse without special treatment. They were ex-

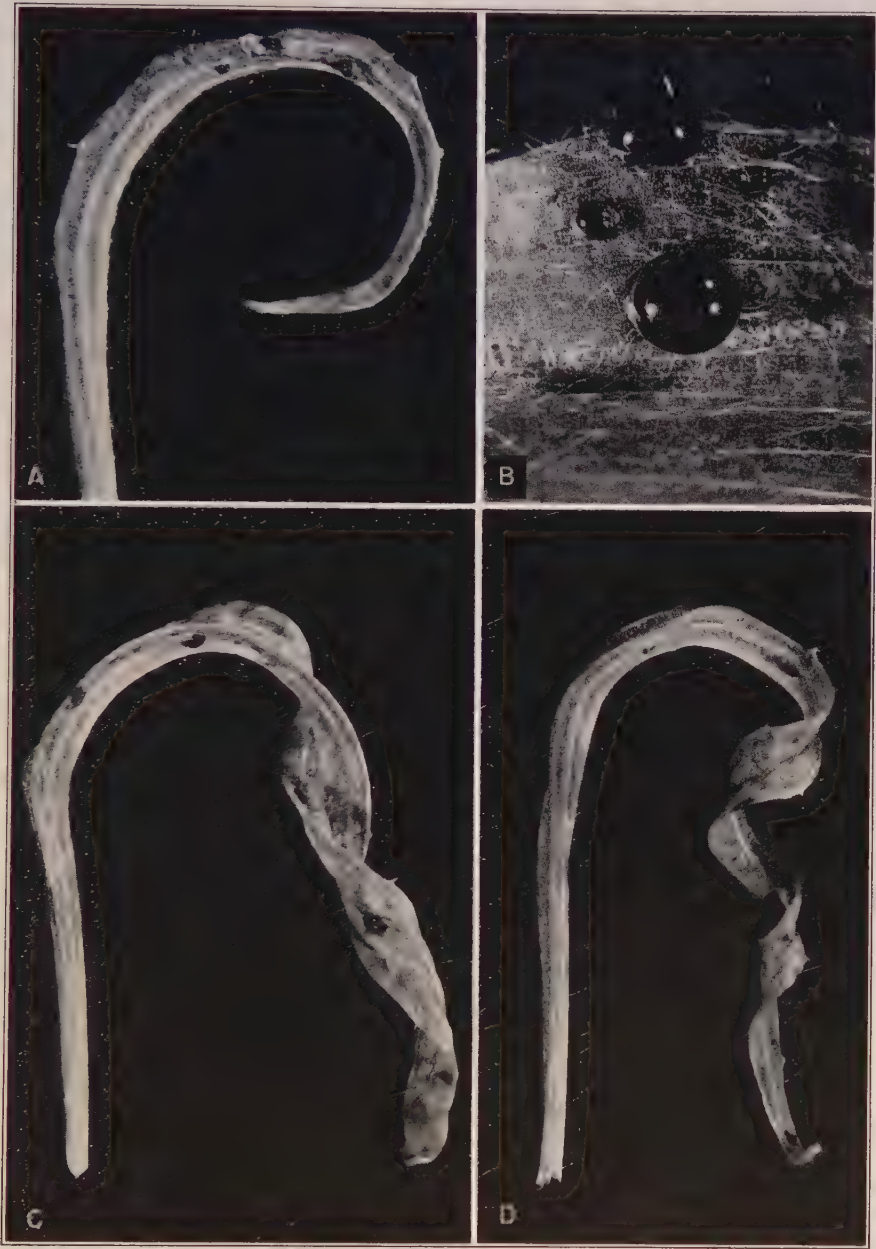


FIGURE 1. Exudate on tetraploid leaves of *Plantago ovata*. (A, C, D) Distorted leaves with drops of exudate which occur on both upper and lower epidermal layers, $\times 1.0$. (B) Exudate on the upper epidermis, $\times 7.5$.



FIGURE 2. Photomicrographs of cross sections of *Plantago ovata* leaves: (A) diploid, (B) tetraploid, (C-F) tetraploids after exudate appears on leaves. Frozen sections 75μ thick. (A-C, E) $\times 15$. (D, F) $\times 30$.

amined daily for the appearance of macroscopic flower buds or exudate. In September and November plantings diploids began to flower after 84 days, while tetraploids began after 98 days. A February seed planting resulted in flowers 42 and 47 days later for diploids and tetraploids respectively. Data to be presented below show a light relationship here. Exudate formation in these large untreated lots was as follows: of 66 diploid and 62 tetraploid plants in Series 1 planted in September, 6 and 34 per cent showed exudate; of 152 diploid and 105 tetraploid plants in Series 2 planted in November, 13 and 97 per cent showed exudate; and of 457 diploid and 487 tetraploid plants in Series 3 planted in February, 2 and 62 per cent showed exudate.

Special tests to be described below were designed to determine differences in physiological responses of diploids and tetraploids to environmental conditions and to show whether exudate formation is related to type of soil and nutrition, temperature, moisture, light, fumigation, or chemical composition. All plants were grown in the greenhouse in 4-inch pots after first being transplanted from the seed pan to 2-inch pots. Series 1 plants were grown from diploid seeds collected in India in 1942 and from tetraploid seeds collected from colchicine-induced plants grown in the greenhouses at Yonkers, N. Y. For Series 2 and 3, both diploid and tetraploid seeds were collected in March and April, 1954, from plants grown at Yonkers, N. Y.

NUTRITION EFFECTS

Seeds of both diploids and tetraploids were planted on November 2, 1953. Fifteen days later, seedlings were transplanted into 4-inch pots in three different types of soil. Soil 1 was a mixture of granulated peat moss, river sand, and sterilized loam in equal parts; soil 2 was a very infertile, compact sod soil; and soil 3 was composed of four parts sterilized loam, two parts leaf mold, and one part river sand with fertilizer added. Plants previously grown in soil 3 had produced the exudate.

Twenty-five or more plants each in the three types of soil received no further treatment while others were subjected to eight different conditions to be described below. Combining all conditions, each type of soil was represented by at least 225 diploid and the same number of tetraploid plants. Summarizing the soil effects regardless of other treatment, plants in soil 3 flowered slightly earlier and completed flowering sooner than those in soil 1, and those in soil 2 lagged far behind those in both 3 and 1. Growth was reduced in soil 1 as compared with that in soil 3. Growth in soil 2 was so poor that many of the plants had failed to flower by the termination of the experiment. The percentage of tetraploids producing exudate was greatest for plants in soil 3 (31 per cent); intermediate in soil 1 (17 per cent); and least in soil 2 (1 per cent). The failure to produce exudate may

have been due to the poor growth in soil 2. Exudate appeared on 4 per cent of a total of 341 diploids and on 30 per cent of 345 tetraploids as shown by combined data from all soils and treatments.

Limited tests were made on plants in nutrient solution and in sand watered with nutrient solutions. In addition to a balanced nutrient, solutions with deficiencies of potassium, phosphorus, or nitrogen and with an excess of nitrogen were prepared according to the methods described by Loomis and Shull (10, p. 62). Plants in liquid and sand cultures were grown in the greenhouse for a period of one month when they were discarded because of poor growth. There was no apparent relation between nutrient supply and the appearance of exudate.

TEMPERATURE EFFECTS

Lots of 25 each of diploid and tetraploid plants in the three types of soil described above were transferred to 5°, 10°, or 30° C. each night (16 hours) and returned to the greenhouse during the day (8 hours). Plants kept in the greenhouse with a night temperature of 21° C. served as controls. It should be pointed out that the photoperiod for plants given 5°, 10°, and 30° C. at night was only 8 hours per day while the greenhouse controls received 9 hours of light per day at the beginning of the experiment (Dec. 30) and 12 hours per day at the termination of the experiment on March 24. It will be shown below that a photoperiod of 8 hours retards flowering. In spite of light differences, however, certain differences in temperature effects were noted.

Diploid plants receiving 30° C. at night flowered 2 days earlier than the controls, but made poorer vegetative growth than either the controls or those receiving low temperature at night. The first flowers on tetraploid plants also appeared on those receiving 30° C. night temperatures and were 9 days ahead of the first flowers on the controls. However, very few flowers were produced under these conditions, while the controls flowered abundantly. Plants of both diploid and tetraploid at low night temperatures, especially 5° C., were delayed in flowering as much as a month. Whereas diploid controls always formed flowers one to two weeks sooner than the tetraploids, both lots flowered at the same time after exposure to low night temperatures. Exposure to special night temperatures was continued for 84 days after planting.

Low night temperatures resulted in good vegetative growth and final production of good seeds for both diploid and tetraploid plants. No differences between diploids and tetraploids in tolerance to different temperatures were noted and exudate formation did not appear to depend on temperature. However, high night temperature reduced growth and number of flowers of all plants and might well be a factor in the growth of this species of *Plantago* for psyllium production in this country.

To study further the effect of low temperature on the growth and reproduction of *Plantago*, a second series of plants were exposed to 5° C. for 1, 3, and 7 days after which they were returned to the greenhouse. This treatment was given to seedlings at 1, 2, 4, and 6 weeks of age. Seven days at 5° C. lowered the survival of seedlings of both forms and all ages, but 1 and 3 days had little effect. Again flowering was delayed over that of the controls, but exudate formation was not affected by the low temperature.

WATER RELATIONS

The three different soil types described above were watered normally or excessively for plants under several different conditions so that finally more than 300 pots each of diploids and tetraploids were involved. Within the limits of this test, moisture variation did not affect the rate of flowering. Also, the percentage of plants with exudate was, perhaps, not significantly affected even though tetraploid plants kept wet showed a slightly higher percentage of exudate throughout. Excessive watering caused a slight chlorotic condition—a yellow-green as compared to a much darker green in comparable plants held drier—of both diploid and tetraploid plants receiving a night temperature of 5° or 10° C.

In another series, soil 3 was used for seedlings which received scant, normal or excessive watering. Limited water supply decreased the growth of both diploid and tetraploid and affected flowering. Diploid plants kept dry were smaller, but flowered earlier and had more flowers; those receiving an intermediate amount or an excess of water were larger but with fewer, later flowers (Fig. 3 A). Tetraploid flowering was hastened by 8 days as a result of dry conditions; best vegetative growth was obtained with an intermediate amount of water, but flowering was delayed; and there was definite evidence of injury by excess water (Fig. 3 B). The leaves of tetraploids which were watered heavily became brittle and very much curled, those watered lightly had little curling, and those with medium watering showed medium curling. There was very little curling in corresponding diploid leaves (Fig. 3 A). It will be recalled that curled leaves are characteristic of those producing exudate (3), and that, in an earlier series, tetraploid plants receiving excess water showed a somewhat higher incidence of exudate. Such was not the case in this series, however, since 100 per cent of the tetraploids in this special watering experiment developed the exudate, regardless of the amount of water supplied.

Humidity effects. Plants were enclosed in humidity cages in the greenhouse from December 29 to February 25. The cages were 33×33×36 inches. They were covered on three sides by vinylite (.003 inch) and were placed over the plants which were on 6-inch boards with cracks between them. It was found that the high humidity favored infection by *Botrytis*, delayed flowering and prevented exudate formation. However, some en-



FIGURE 3. (A) Diploid and (B) tetraploid plants of *Plantago ovata* receiving (left to right) light, medium, and heavy watering.

tire leaves of tetraploids in the humid cages had internal browning.

Transpiration. For transpiration experiments, 10 plants each of diploid and tetraploid seedlings growing normally in soil 3 were used. Plants were 68 days old from seed, with no flower buds visible. The soil was watered thoroughly at the beginning of the test and no more water was added during the test. The pots and soil were covered with aluminum foil after wrapping the leaves at soil level with cotton to prevent injury. Weights of the individual pots, soil and plants were taken twice daily (8:30 a.m. and 3:30 p.m.) for 4 days. At the termination of the experiment, the plant

parts above ground were harvested and fresh weights taken. The areas of the leaves of each plant were then determined by the use of a planimeter on tracings made of the individual leaves, after which the leaves were dried and the dry weights recorded. It will be noted (Table I) that diploid plants had an average of 27.8 leaves each while the tetraploid had 15.6. There was great variation in leaf numbers and weights among the 10 plants of each type. The 10 diploid plants showed a range in leaf number from 15 to 45, having fresh weights from 12.45 to 24.45 g. while the tetraploid

TABLE I
WATER LOSS IN 4 DAYS FROM 10 PLANTS EACH OF DIPLOID AND TETRAPLOID
PLANTAGO OVATA GROWING IN SOIL

| Plants | Leaves | | | Water transpired (grams) | | | | |
|------------|--------|-------------------|-----------------|--------------------------|----------|---------|---------------|------------------|
| | Number | Area (sq. in.) | Dry wt. (g.) | Total | G./plant | G./leaf | G./sq. in. | G./g. dry wt. |
| Diploid | 278 | 182.10 | 3.046 | 445.2 | 44.52 | 1.60 | 2.44 | 146.15 |
| Tetraploid | 156 | 123.05 | 2.4596 | 339.0 | 33.90 | 2.17 | 2.75 | 137.82 |

numbers ranged from 9 to 29, and fresh weights from 8.2 to 22.3 g. Total areas and dry weights of the diploid leaves from 10 plants exceeded those for the tetraploids. The total water transpired as well as the amount per plant and per gram of dry weight was less for the tetraploid than for the diploid. However, transpiration per leaf or per square inch of leaf surface was greater in the tetraploid.

Osmotic pressure. The osmotic pressures of the cell sap of diploid and tetraploid plants were compared by both plasmolytic and cryoscopic methods. Freezing point depression measurements were made on sap expressed from the green portion of the plants. Before cutting the plants at soil level, the pots in which they were growing were set in water until the soil was thoroughly soaked (about 45 minutes). After cutting, the plants were transferred to stoppered Pyrex tubes and fresh weights taken. Then they were frozen for 30 minutes in a methanol-dry ice bath and placed in a food freezer at -18° C. overnight. The tissues were then allowed to thaw before placing in a canvas wrapping under the hydraulic press, where a pressure of 3080 pounds per square inch was sustained for one minute. Using a Beckman thermometer, freezing points were then determined on the expressed sap and these were compared with that of distilled water. From the depression of the freezing point, osmotic pressures were calculated according to the method of Harris and Gortner (5).

Sap was expressed from diploid and tetraploid seedlings 21, 32, 36, and 54 days after planting. The fresh weight of the green tissue and the volume of sap expressed together with the osmotic pressures in atmospheres deter-

mined by freezing point depression are shown in Table II. Averages of duplicate readings for osmotic pressure are given. It should be pointed out that the 21-day-old seedlings were very small and in poor condition in the original seed pan. Their poor growth and low moisture content are reflected in a higher osmotic pressure. After 32 and 36 days, plants growing normally in pots were in healthy vegetative growth. Plants showing macroscopic flower buds were chosen for testing after 54 days. In every case, the osmotic pressure for the diploid exceeded that of the corresponding tetraploid.

Plasmolytic determinations were made on the 32-day plants by stripping the upper epidermis from the leaf. Sections of epidermis were mounted

TABLE II
CHANGES IN OSMOTIC PRESSURE OF SAP FROM DIPLOID AND TETRAPLOID *PLANTAGO OVATA* SEEDLINGS WITH AGE

| Age of plants (days) | Diploid or tetraploid | Seedlings | | Sap expressed (ml.) | Osmotic pressure (atm.) |
|----------------------|-----------------------|-----------|----------------|---------------------|-------------------------|
| | | No. | Fresh wt. (g.) | | |
| 21* | D | 75 | 4.8730 | 2.4 | 23.4 |
| | T | 75 | 5.1554 | 3.6 | 14.3 |
| 32 | D | 10 | 5.5256 | 2.5 | 20.5 |
| | T | 10 | 8.0142 | 4.3 | 10.9 |
| 36 | D | 10 | 10.7606 | 6.5 | 15.7 |
| | T | 10 | 13.8342 | 9.5 | 9.2 |
| 54 | D | 5 | 23.3404 | 13.8 | 20.3 |
| | T | 5 | 21.3878 | 14.0 | 8.4 |

* In seed pans.

in 0.05 *M* sucrose solution. Cytoplasm of cells of both diploid and tetraploid plants remained turgid. With filter paper various molar concentrations (0.1, 0.2, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 2.0) of sucrose were drawn under the cover glass of the mounted epidermis. The inception of plasmolysis in the diploid was observed in 0.8 *M* concentration while 0.2 *M* initiated plasmolysis in the tetraploid. These tests were repeated 4 days later with similar results. No plasmolysis was obtained in the diploid with 0.75 *M* while 0.8 *M* concentration gave slight plasmolysis. Since 0.2 *M* sucrose previously gave slight plasmolysis in tetraploid cells, 0.15 *M* solution was tested on the tetraploid but gave no plasmolysis. These values represent an even greater difference in solute concentration in the cell sap between diploid and tetraploid than that shown by freezing point depression, and leave no doubt that the solute concentration of the cell sap of the diploid is at least 1.5 times that of the tetraploid. This may be accounted for in large part by the higher moisture content of the latter in proportion to the dry weight (Table III).

LIGHT RELATIONS

Since more exudate has appeared on plants grown during the winter months, an experiment to determine the possible effect of light was initiated. Seeds were planted on February 2, 1955. On February 8 they were transplanted into 2-inch pots, and on February 14 they were placed under various light conditions. They were transplanted further into 4-inch pots at a later date. Fifty plants each of diploid and tetraploid were placed under short-, normal-, and long-day conditions. The first was achieved in the greenhouse by covering with a cage (covered with black cloth) each

TABLE III
MATERIAL USED FOR EXTRACTS OF DIPLOID (D) AND TETRAPLOID (T) *PLANTAGO*
OVATA FOR CHROMATOGRAPHIC ANALYSES

| Material | Age (days) | Num- ber | Fresh wt. (g.) | | % Moisture (fresh wt. basis) | | % Moisture (dry wt. basis) | |
|-----------------|---------------|-------------|----------------|------|---------------------------------|----|-------------------------------|------|
| | | | D | T | D | T | D | T |
| Mature seeds | — | 1000 | 1.7 | 2.8 | — | — | — | — |
| Green seedlings | 5 | 100 | 1.5 | 2.2 | — | — | — | — |
| | 12 | 100 | 3.0 | 4.5 | — | — | — | — |
| | 15 | 10 | 1.2 | 1.8 | 93 | 94 | 1431 | 1680 |
| | 28 | 10 | 4.8 | 5.3 | 93 | 94 | 1427 | 1704 |
| | 42 | 5 | 14.6 | 13.9 | 91 | 93 | 961 | 1341 |
| | 56 | 2 | 7.3 | 12.7 | 90 | 93 | 875 | 1310 |
| | 70 | 2 | 11.0 | 17.9 | 87 | 91 | 670 | 1052 |

day at 5 p.m. and removing the cage the next morning at 8 a.m. (8-hour photoperiod). Normal daylength was that present at this season (10 hours Feb. 14 to 14 hours May 10). Long-day conditions were achieved by two 500-watt Mazda lamps placed 2 feet above the plants. These lamps were turned on at 5 p.m. and off at 10 p.m. daily, an average extension of the normal photoperiod by about 4 hours. The experiment was terminated on May 10.

The daylength had a definite effect on the flowering response of both diploid and tetraploid plants. In the case of the diploid, the first flowers appeared under long-day conditions 30 days after planting. Ninety per cent of these plants had flowered in 37 days and 100 per cent in 44 days. Flowering under normal daylength began after 42 days and was complete in 58 days, a delay of 12 and 14 days. Under short-day conditions flowering of the diploid did not begin until 61 days after planting and was complete after 77 days, a further delay of 19 days for beginning and termination of the flowering period over that of plants under normal daylength. Some of these effects are shown in Figure 4 A which pictures diploid plants 59 days after planting.

The effect of daylength on the flowering of tetraploids is shown in Figure 4 B and is graphically represented in Figure 5. The first flowers in the

long-, normal-, and short-day plants appeared after 37, 47, and 61 days with flowering complete under the three conditions after 56, 65, and 75 days. Comparing the diploid with the tetraploid, data show that appearance of the first flower of the latter was 7 and 5 days later under long and normal daylengths, but both forms flowered at the same time under short periods of light.

Day-by-day records of the appearance of exudate were kept to determine whether daylength affected it in any way; especially to see whether there was any correlation between the onset of flowering and the appearance of the exudate. Exudate appeared on only two of the entire lot of diploid plants in this series. The total percentages of tetraploid plants producing exudate under long-, normal-, or short-day conditions were 51, 70, and 92 respectively. This may, again, point to a moisture relationship, since



FIGURE 4. (A) Diploid and (B) tetraploid plants of *Plantago ovata* receiving (left to right) short, normal, and long photoperiods.

less water would be transpired under short periods of light. Three and 9 per cent of the total number of plants affected had produced the exudate after 37 days in normal and short days respectively. These percentages had increased up to 15 and 21 by the 47th day when the first exudate was found on the plants grown under long-day conditions. This may indicate that a condition causing a delay in the maturity of the plant hastens the

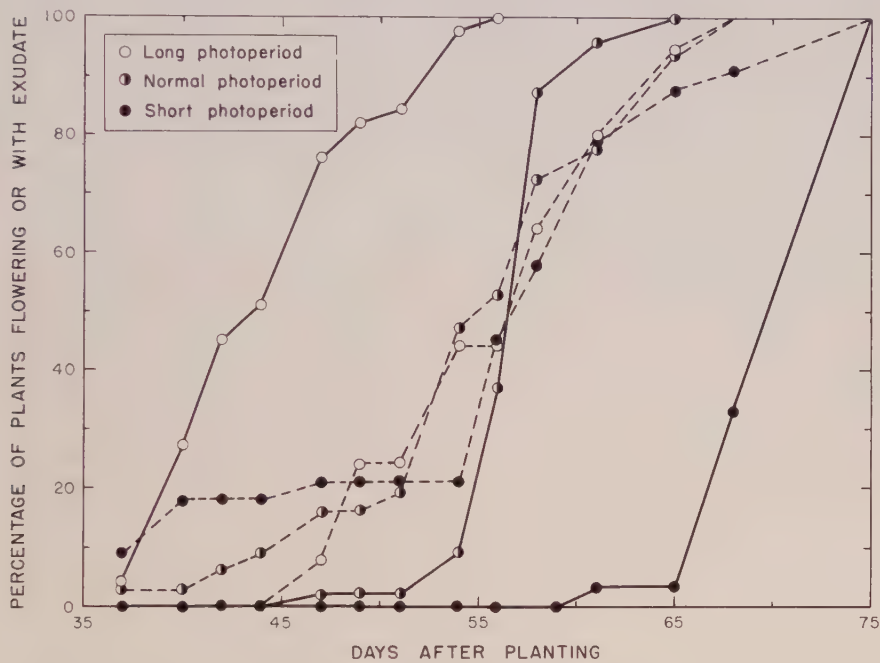


FIGURE 5. Time of flowering and exudate formation in tetraploid plants of *Plantago ovata* receiving long, normal, and short photoperiods. Percentage of plants flowering is represented by solid lines and percentage of plants with exudate is represented by dotted lines.

appearance of the exudate. However, further progress of the exudate in number of plants affected was about the same for all light exposures.

Graphs of the exudate production under the three daylengths together with curves for flower formation under the same light conditions are shown in Figure 5. Exudate formation appears to have been hastened by exposure to a short photoperiod. In the interval from 37 to 47 days after planting, more exudate was produced by the tetraploid plants under short photoperiod than by those under normal light conditions. On the other hand, a long photoperiod, which hastened flowering, seemed to delay exudate formation. However, it is obvious that the degree of maturity of the plants has no sustained effect on exudate formation. For example, 20 to 64 per cent of all plants with exudate under any daylength were affected in

the interval from 49 to 58 days. All of this development occurred after the plants under long-day conditions had macroscopic flower buds and before those under other light conditions had bloomed. Further exudate formation occurred after plants under normal daylength had bloomed but before those in short daylength had flowered. It appears that either the age of the plant or some environmental condition, other than light, prevailing in the greenhouse determined the time of formation of the exudate.

To test this hypothesis further, plantings of both diploid and tetraploid seeds were made in the greenhouse at weekly intervals for four weeks; April 20 (A), April 27 (B), May 4 (C), and May 11 (D). Flowering and exudate formation on a total of 194 diploid and 190 tetraploid plants thus produced were noted. Again, there was no clear correlation between the time of flowering and the appearance of the exudate. However, the number of tetraploids producing the exudate varied greatly. Sixty-nine per cent of the plants from seed planting A, and 60 per cent of those from planting B developed the exudate. Planting C showed a reduction to 49 per cent while planting D was remarkably free of the exudate (9 per cent). All of these plants were grown under normal daylength which was about 13 hours on April 20, and which increased to 15 hours on June 21, and then decreased to 14 hours on July 25 when the experiment was terminated. It is possible that daylength is critical in the early vegetative stages of the plant. Also, it should be noted that most of planting D reached maturity the first part of July, when very high temperatures doubtless increased the transpiration rate. The first three plantings had passed the peak of flowering and exudate formation before the advent of very hot weather. Diploids, normally without exudate, showed the same trend as the tetraploids. Percentages of these plants on which exudate was noted were 14, 6, 3, and 0 for plantings A, B, C, and D respectively.

FUMIGATION EFFECTS

Since both cyanide and nicotine fumigation had been used routinely in the greenhouse for the control of insects on *Plantago*, it seemed worth while to determine whether possible injury by these chemicals could account for exudate formation. Consequently, greenhouses containing diploid and tetraploid plants were treated with cyanogas (Virginia-Carolina Chemical Co., Lancaster, Pa.) or Nico-Fume liquid (American Cyanamid Co., New York, N. Y.) at recommended dosages. Other plants were treated by the two fumigants in succession. Cyanogas treatment caused a considerable amount of injury to the young leaves in the center of both diploids and tetraploids. Older leaves were not so affected. Nicotine treatment caused no apparent injury when applied alone or after cyanogas treatment. There was no clear relation between these treatments and flowering or exudate formation. However, cyanogas treatment appeared to de-

lay flowering, especially in the diploid. Also the earliest exudate appeared on tetraploids treated with cyanogas, but neither the time of appearance nor the total number of plants affected differed significantly from the other treatments.

All plants were particularly susceptible to aphid infestation, which was heavy in plants which received no fumigation, i.e. the control lots. Therefore, the possibility that aphid injury of the controls may have offset any fumigant injury should not be overlooked.

CHROMATOGRAPHY

Methods

Paper chromatography was employed as a method of determining the differences in free amino acids and sugars (1) in seedlings of diploid and tetraploid *Plantago* at various stages of development. After some experimentation to determine a method for preparing a satisfactory extract for spotting chromatograms the following procedure was adopted.

Plants were severed at the surface of the soil, and the tissue was cut into small pieces and homogenized with 80 per cent ethanol in a VirTis "45" homogenizer.¹ The homogenate was quantitatively transferred to centrifuge containers, using 80 per cent alcohol for washing. Centrifugation was for 15 minutes in an International #2 centrifuge² in a 100-ml. tube at 1315 R.C.F. (relative centrifugal force). After decantation, the residue was washed with 80 per cent ethanol until the green color was gone. The total volume of 80 per cent ethanol used was at least 8 to 10 times the green weight of the tissue. The ethanol was then evaporated, and the residue taken up in chloroform and water (first one and then the other, then a mixture of the two) and transferred quantitatively to a 50-ml. centrifuge tube and evaporated to dryness. Six ml. of chloroform and 10 ml. of water were added to the residue and centrifuged for 15 minutes at 2050 R.C.F. Nine ml. of the aqueous phase were then removed and taken to dryness. The residue was taken up in a known volume of water and used to spot chromatograms.

Extracts were made of dry seeds and of green seedlings 5, 12, 15, 28, 42, 56, and 70 days after planting. The amount of comparable diploid and tetraploid material used in each case is shown in Table III. Also shown in this table are moisture percentages of samples taken at the same time as those extracted. These percentages have been calculated on both fresh and dry weight bases. Data on the chromatographic analyses will be given below on the basis of the fresh weight of the plant tissues. However, it should be noted that, although moisture percentages of diploid and tetra-

¹ The VirTis Company, Inc., Yonkers, N. Y.

² International Equipment Co., Boston, Mass.

ploid tissues were about the same when calculated on the basis of fresh weights, those of tetraploids were much higher than diploids on a dry weight basis. This means that for a given green weight, more diploid tissue was represented.

The exudate was collected from the leaves with a hypodermic syringe and applied directly to the chromatograms. An effort was made to get a quantitative measurement by washing the exudate from the syringe with distilled water into a weighed vial, and placing in an evacuated desiccator over concentrated sulfuric acid to dry. Finally, a dry weight of 143 μ g. was represented in a 1- μ l. spot. However, because of the possible variation in the exudate at different times of collection, only qualitative results of these chromatograms will be given.

Ascending two-dimensional chromatography was used throughout to estimate free amino acid content of the tissues. Spotted papers (Whatman No. 1) were placed in a trough containing liquid 88 per cent phenol and distilled water (100:20 v/v) and left until the liquid front reached the top of the papers, usually 5 to 6 hours. The papers were then dried overnight in a hood with a strong draft. The following morning they were placed in a mixture of lutidine-100 per cent ethanol-distilled water-diethylamine (55:20:25:1 v/v) and again left until the liquid front reached the top, and dried overnight in the hood. The papers were then dipped in ether to remove any blue color due to the presence of diethylamine. After drying they were dipped in ninhydrin (0.2 per cent in acetone), and developed at 35° C. for 90 minutes.

Solvents tried for sugar chromatograms were: 1) butanol-acetic acid-water (40:10:50); 2) ethyl acetate-pyridine-water (2:1:2), organic phase; 3) phenol-water (80:20); and 4) isopropyl alcohol-58 per cent ammonium hydroxide-water (80:5:15). The last named proved satisfactory for sugars and was used for estimation of these substances in the plant extracts.

Glucose, the principal sugar found in *Plantago* extracts, was detected by spraying with a solution made by mixing 4.98 g. phthalic anhydride, 2.73 ml. aniline, and 300 ml. 100 per cent ethanol. For other sugars, 0.2 per cent naphthoresorcinol (w/v) in ethanol, and 2.0 per cent trichloroacetic acid (w/v) in water were mixed in equal volumes just before using. Papers were dried in the hood at room temperature and then heated at 100° C. for five minutes.

Approximate quantitative determinations of amino acids were made by measuring optical densities of the spots by means of a Photovolt electronic densitometer, consisting of a Standard Transmission Density Unit Model 52-C and α Multiplier Photometer Model 520-M.³ Each spot was searched using a circular aperture 1 mm. in diameter and the maximum density of

³ Photovolt Corporation, New York, N. Y.

the spot was recorded. Filter 550 was used for the amino acids visible on the paper as blue or violet spots. For asparagine, which forms a brown color on treatment with ninhydrin, filter 440 was used.

Results

Amino acids. Diploid and tetraploid tissues were compared in their contents of free amino acids. Values for standards to cover the quantitative range for each acid and each tissue were included in each run, and the amino acid values for each run were calculated from its standards. Standards were in replicates of four and extracts in duplicate. Two complete series were run.

Data from the second series are shown in Table IV. Diploid and tetraploid seeds and seedlings contained the same free amino acids and in approximately the same quantities per 100 mg. of fresh tissue. The relatively large amounts of asparagine and aspartic acid are probably of some

TABLE IV
AMINO ACID AND AUCUBIN CONTENT OF EXTRACTS OF SEEDS AND GREEN SEEDLINGS OF DIPLOID AND TETRAPLOID *PLANTAGO OVATA*

| μg. Chemical per 100 mg. fresh tissue | | | | | | | | | | | | | | | | |
|---------------------------------------|-----------|-------------------------|----|----|----|-----|-----|----|------------|-------------------------|----|----|----|----|-----|----|
| Chemical | Diploid | | | | | | | | Tetraploid | | | | | | | |
| | Dry Seeds | Age of seedlings (days) | | | | | | | Dry Seeds | Age of seedlings (days) | | | | | | |
| | | 5 | 12 | 15 | 28 | 42 | 56 | 70 | | 5 | 12 | 15 | 28 | 42 | 56 | 70 |
| Alanine | 3 | 6 | 5 | 5 | 4 | 5 | 7 | 2 | 1 | 5 | 3 | 6 | 3 | 5 | 9 | 4 |
| γ-Aminobutyric acid | 0 | 0 | 0 | 0 | 1 | 2 | 4 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 4 | 2 |
| Arginine | 0 | 13 | 4 | 5 | 2 | 2 | 0 | 0 | 0 | 10 | 3 | 4 | 1 | 2 | T | 0 |
| Asparagine | 23 | 44 | 15 | 14 | 5 | 2 | T | 5 | 7 | 80 | 16 | 8 | 4 | 3 | T | 3 |
| Aspartic acid | 22 | 12 | 7 | 26 | 17 | 12 | 4 | 2 | 5 | 8 | 4 | 4 | 12 | 10 | 1 | 1 |
| Glutamic acid | 11 | 19 | 13 | 6 | 2 | 7 | 11 | 4 | 15 | 10 | 11 | 3 | 9 | 16 | 3 | 3 |
| Glutamine | 1 | 22 | 7 | 9 | 8 | 10 | 16 | 2 | 1 | 28 | 13 | 7 | 7 | 12 | 10 | 4 |
| Leucine | 2 | 0 | 0 | 4 | 3 | 2 | 4 | 1 | 0 | 7 | 3 | 3 | 3 | 2 | 9 | 2 |
| Serine | 6 | 4 | 3 | 4 | 1 | 1 | 2 | 1 | 1 | 4 | 3 | 3 | 1 | 1 | 4 | 1 |
| Threonine | 3 | 0 | 2 | 4 | 1 | 2 | 2 | 1 | 1 | 4 | 2 | 3 | 1 | 1 | 3 | 1 |
| Valine | 1 | 6 | 2 | 4 | 2 | 1 | 2 | 1 | 1 | 6 | 2 | 3 | 2 | 1 | 3 | 1 |
| Aucubin | 31 | 12 | 8 | 23 | 49 | 157 | 127 | 89 | 12 | 15 | 4 | 18 | 36 | 69 | 114 | 57 |

T = trace.

significance; also the small amount of glutamine and the apparent total lack of arginine in the seeds as compared with that present in 5-day-old green seedlings, and subsequent reduction of these amino acids with increased age of the seedlings. γ-Aminobutyric acid was not detectable in seeds or in green seedlings younger than 28 days. An accumulation of salts in the extracts of *Plantago* plants with increasing age after 28 days caused a streaking on the chromatograms in the region of aspartic and glutamic acids resulting in difficulty in reading the optical densities of these spots. Removing these salts by passing the extracts through resin ion exchange columns prevented the streaking but gave lower optical densities; hence these data are not presented here.

Sugars. Because of background color, which always developed, densi-

tometer readings for quantitative estimation of sugars were not reliable. Papers treated with the aniline acid phthalate reagent showed a trace of glucose in 5-, 12-, 15-, and 28-day seedlings with an increased amount in 42-day and much larger amounts in 65- and 70-day plants. When compared visually, tetraploid seedlings seemed to contain more glucose than the diploid. This was especially marked at 56 and 70 days. Papers treated with the naphthoresorcinol reagent showed essentially the same relative amounts of sucrose and sorbose in the extracts of the seedlings of different ages as demonstrated for glucose, though the latter appeared to be in greater quantity.

Exudate. Whether fresh or dried exudate was used to spot chromatograms, strong concentrations of amino acids were indicated. In addition to all of those shown in Table IV for the plant extracts lysine, tyrosine, and probably histidine were present in the exudate. Also the quantity of arginine appeared to be greater in the exudate than in the tetraploid plant extract. There was a striking increase in the number and quantity of amino acids in the exudate of the tetraploid plant as compared to the mature tissues from which the exudate was produced. This may indicate the digestion of the tissues in the process of exudate formation.

The exudate also contained sugars, chief among which was glucose. Sucrose was present, and possibly also raffinose and fructose.

Fresh exudate chromatograms developed with isopropyl alcohol-58 per cent ammonium hydroxide-water solvent and sprayed with Ehrlich's reagent (1 per cent solution of *p*-dimethylaminobenzaldehyde in *N* HCl) showed the presence of large amounts of tryptophane but no indole-3-acetic acid. A pale lemon-yellow spot appeared at *R_f* .08, and a pinkish-yellow spot at *R_f* .94, also a very dark, greenish-gray spot at *R_f* .55. This last spot was shown later to be the glycoside, aucubin.

Aucubin. A reference to published literature revealed that the glycoside, aucubin, had been discovered by Bourquelot and Hérissé in 1905 in tissues of *Aucuba japonica* L. (2) and had been found to yield a brown color on oxidation. Trim and Hill (13) reported the same substance in unripe fruit of *Plantago lanceolata* L.

Using green tissues (1200 g. diploid; 1125 g. tetraploid) of old plants after seeds had been collected and the method of Trim and Hill (13), a partially purified form of aucubin was obtained in this laboratory. Upon chromatographing, it was found that it occupied the same position and gave the same color as the dark, greenish-gray spot already detected on a chromatogram of the exudate. Then, through the courtesy of R. Mavrodineanu of this Institute, a sample of the original lot of aucubin discovered by Bourquelot and Hérissé (2) was obtained from Prof. M. M. Janot, Laboratoire de Pharmacie Galénique, Paris, France. These pure crystals were compared with the preparation made in these laboratories

and with the unknown in the exudate. They appeared to be identical. The substance was further identified by using four of the solvents recommended by Janot, Sañas and Foucher (8) for paper chromatography of aucubin, with the result that corresponding Rf values were found. Although the first aucubin spots were detected in the exudate of tetraploid *Plantago ovata* by spraying with Ehrlich's reagent, they are also stained by naphthoresorcinol reagent spray used for sugars. Aucubin disappeared from the exudate when it was dried or stored in water solution.

Having demonstrated the presence of aucubin in the brown exudate of tetraploid *Plantago ovata*, and in the extract of old *Plantago ovata* plants, it became of interest to determine whether both diploid and tetraploid plants possessed this glycoside in equal quantities. Consequently, one-dimensional chromatograms were prepared using all of the extracts shown in Table III, with standards of the Paris aucubin in concentrations of 0.28, 0.14, 0.07, 0.035, and 0.0175 per cent in distilled water. Six replicates of the standards in spots of 10 μ l. each were used. Plant extracts and exudate preparations were in replicates of three in applications of 5, 10, and 20 μ l. each. Isopropyl alcohol-58 per cent ammonium hydroxide-water was used as the solvent and Ehrlich's reagent for detection. Readings were made on the Photovolt densitometer using the 550 filter. Two complete series were run, with essentially the same results. Data obtained from one of these are shown in Table IV. It will be noted that the diploid contained, in general, more aucubin per 100 mg. of green tissue than the tetraploid, though these differences are probably not significant in view of the lower dry weight of the tetraploid (Table III). However, both diploid and tetraploid showed the same trend in amounts present at different stages of development of the seedling. At least half of the glycoside present in the seed seemed to disappear by the time the seedling was 12 days old. After that time the amount increased at a rapid rate until 42-day-old diploid seedlings contained almost 20 times as much as 12-day-old seedlings and 5 times as much as was present in the seed. This peak came 14 days later for the tetraploid but the increase was of the same order of magnitude. It will be recalled that the tetraploid matures more slowly than the diploid plant and this may account for the delay in aucubin production, since the maximum amount appears to be produced about the time of flowering. Diploids and tetraploids in this series had macroscopic flower buds 42 and 47 days after planting.

An attempt was made to determine the distribution of aucubin inside the tissue of a normal leaf. When the entire leaf was placed over concentrated hydrochloric acid, it lost its green coloring and a dark brown precipitate was apparent in the palisade and parenchyma cells when the tissue was macerated and examined under a microscope. This indicated the presence of a material similar to the exudate. Further indication that

this precipitate was due to aucubin in the cells was obtained by an adaptation of a procedure given by Tunmann and Rosenthaler (14, p. 640) for obtaining and testing aucubin from plants. Sections of leaves were cut into pieces, placed in water and boiled gently for 15 minutes to extract aucubin. The liquid was decanted and 7 drops of concentrated hydrochloric acid added. After 20 to 30 minutes, the liquid had a blue-green color which gradually intensified to blue-violet, a characteristic reaction for aucubin. Treatment of this boiled tissue with hydrochloric acid failed to reveal a brown precipitate in the cells thus indicating that the brown color noted above was probably due to the presence of aucubin, removed, in this case, by boiling. These tests did not indicate the presence of aucubin in normal epidermal cells, though, of course, its presence could be demonstrated there after the sap had exuded to the surface of the leaf (see MORPHOLOGY above).

DISCUSSION

A general review of the literature on the physiology of polyploidy in plants has been made by Noggle (11). There seems to be a general agreement that polyploidy brings about a slower rate of growth so that tetraploids are two days to a week behind the diploids in physiological development. This was found to be true for *Plantago ovata* used in this study.

A search for the cause of a brown exudation, which appears especially on tetraploid *Plantago ovata* leaves, has been made in the comparison of several physiological responses and the chemical content of the diploid and colchicine-induced tetraploid forms.

The color of the exudate has been found to be due to the presence in the sap of the glycoside, aucubin. Aucubin may be hydrolyzed by an enzyme (6) or by acid. Upon hydrolysis it yields 41 to 42 per cent aucubigenin and 53 to 55 per cent glucose (2). Aucubin is easily degraded by acid and cannot be kept for long in acid solutions. Hydrolytic enzyme action can be the main cause of the losses and if left to act unhindered, as they would do once the tissues had been injured, these enzymes would rapidly hydrolyze the whole of the available glycoside. The hydrolysis apparently takes place inside the leaf of *Plantago ovata* for the brown color signifying a breakdown product of aucubigenin (2) is inside as well as on the surface of the leaf.

Although the amount of aucubin was increased many-fold during the development of the *Plantago ovata* seedling from 5 to 42 days and reached a maximum at about the time of maximum exudate formation, there was no relation between the amounts present in diploid and tetraploid tissue to account for the higher incidence of exudate in the latter.

Neither the exudate itself nor the deterioration of the leaf tissues following its appearance seemed to be associated with mechanical or insect injury. Many plants severely infested with aphids had no exudate, while

others fairly free of aphids developed large drops of the exudate. Also no clear relation was found between fumigation injury and exudate formation. Examination of the root and stem tissues of affected plants failed to reveal bacterial or fungal infection associated with the exudate. Furthermore, the condition is not transmitted from one plant to another by "inoculation" with the exudate.

Within the limits of the present tests, nutrition, temperature, and light appeared to have no direct relation to exudate formation, though they were not without effect on vegetative growth and flowering.

Differences were found, however, in the water relations of the plants which may account, at least in part, for the greater susceptibility of the tetraploids to exudate formation. Excess moisture in the soil was more harmful to tetraploids than to diploids, causing characteristic curling of the leaves of the former. The osmotic pressure of the cell sap of the tetraploids was considerably less than that of the diploids. Moisture content of the tetraploid green tissues, calculated on the basis of dry weight, was much higher than that of the diploid. This extra moisture may account for the greater part of the decreased osmotic pressure. Most of the experimental evidence presented by other workers (11) indicates that polyploid plants have a higher water content and lower osmotic pressure than diploids. Noggle (11) points out that most of these samples have been taken at only one stage in the development of the plant. Present tests have included seedlings from 15 to 70 days old with results which corroborate the general findings of others. The stem epidermis of polyploid *Pinus sylvestris* has been reported to show low osmotic pressure (6 to 7 atmospheres against normal 9 to 10) and to possess less elastic and more fragile cell wall structure (9). Chen and Tang (4), on the other hand, found the osmotic pressure of colchicine-induced autotetraploid barley higher than in the diploids and Ito and Yamamoto (7) reported the osmotic pressure of the cell sap of diploid radish the same as the tetraploid.

The lower osmotic pressure in young twigs of apple has been regarded by Sokolov and Sokolova (12) as conducive to attacks by aphids. This relationship was not noted in the present tests with *Plantago*.

Chen and Tang (4) measured the water loss by barley plants in a period of 10 hours and found 0.38 and 0.26 ml. per sq. cm. of leaf area for diploid and colchicine-induced autotetraploid plants respectively. This difference was more apparent when the rate was expressed in terms of ml. transpired per unit dry weight. Respective values in the latter case were 78.3 and 38.8 mg./g. The transpiration rate of polyploid *Pinus sylvestris* has been reported as 32 per cent higher than normal (9). In the present tests, the total amount of water transpired in a given period, as well as the amount per plant and per gram of dry weight, was less for the tetraploid than for the diploid. However, the transpiration per leaf or per square inch of leaf area

was greater in the tetraploid. Low transpiration rates coupled with low osmotic pressure might be expected to favor exudation of the cell sap.

Diploid and tetraploid plants have been found by other workers to vary in nitrogen, sugar, and growth substance contents (11). The present tests, using chromatographic procedures, revealed no significant differences between diploid and tetraploid *Plantago ovata* in contents of free amino acids or sugars at stages of development from seed to maturity. However, quantities of amino acids and the glycoside, aucubin, in both diploid and tetraploid were affected by the physiological age of the plants.

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METHOD FOR MEASURING THE CRYSTALLIZATION RATE OF BORDEAUX MIXTURE¹

H. P. BURCHFIELD AND JOAN SCHECHTMAN

SUMMARY

A method is described for measuring the deterioration of Bordeaux mixture through crystal growth which is based on rate of solubilization of copper in solutions of sucrose. The freshly prepared material dissolves rapidly while the aged material dissolves slowly owing to the decreased specific surface of the crystalloid. Soluble copper is determined optically at 650 $m\mu$. Deterioration follows a skewed sigmoid curve with an initial induction period probably corresponding to the time required for nuclei to form. The induction period for 10-10-100 Bordeaux prepared from analytical grade chemicals is 23 minutes at 45° C.

INTRODUCTION

Within a few hours from the time of preparation, 10-10-100 Bordeaux mixture changes from an amorphous hydrogel to a crystalloid (2, 8, 10). It has long been agreed that the mixture should be applied to foliage soon after preparation, for otherwise the *sphaerocrystals* which are formed would not be expected to adhere to plant surfaces when subjected to wind and rainfall owing to their granular nature (1). For the control of Sigatoka disease on banana, 2000-gallon batches are prepared and, if application is delayed because of rainfall, these must sometimes be discarded because of deterioration in the spray tank. It would be desirable, therefore, to have a method available for measuring the extent of crystallization to determine if a mixture is suitable for spraying, and as an aid in developing new formulations with improved stability.

Sedimentation rate (6, 10) and amount of soluble copper in the supernatant (9) have been found to vary with age and conditions of preparation, but it is doubtful if either of these properties can be used to give a true measure of the conversion from hydrogel to crystalloid. However, the observation that sucrose solutions are capable of reacting with Bordeaux mixture to form water-soluble, copper-sugar chelates provides the basis for a quantitative method for measuring the rate and extent of deterioration.

The copper in the amorphous gel will dissolve completely in 10 per cent sucrose within four minutes while the crystalloid will give up only 13 to 14 per cent of its copper in the same length of time, so it is possible to estimate percentage conversion to the crystalloid by a colorimetric measurement of the amount of copper dissolved under these conditions. Subse-

¹ This work was sponsored jointly by the United Fruit Company, Phelps Dodge Refining Corporation, and Standard Fruit and Steamship Company.

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quent publications will show that results obtained by this method correlate with the retentivity of spray deposits in simulated rainfall tests, and also agree qualitatively with crystal growth as determined by X-ray diffraction measurements.

MATERIALS AND METHODS

Chemicals. The present studies were confined to 10-10-100 Bordeaux mixture since this composition is in general use. Analytical reagent grade copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) and calcium hydroxide were used throughout to minimize the effects of traces of impurities on crystallization rate. The cupric sulfate was prepared as a stock solution containing 24 g. per liter. The chelating solution was prepared by dissolving 100 g. of commercial sucrose in 900 ml. of distilled water. It was necessary to make fresh preparations every few days since reducing sugars which interfered with the test were formed slowly due to microbial contamination.

Equipment. Bordeaux mixture for kinetic studies was prepared in a 500-ml., 3-necked flask equipped with a thermometer and sealed stirrer. The flask was immersed in a constant temperature bath regulated at $45^\circ \pm 0.1^\circ$ C., since preliminary experiments showed that the crystallization rate was temperature-dependent, and proceeded too slowly at room temperatures to permit convenient measurement. Samples for analysis were filtered through 30-ml. fritted glass funnels of medium porosity. Filter papers could not be used since they absorbed a considerable amount of copper. The filtrates were collected in test tubes placed in 250-ml. suction flasks. Optical measurements were made with a Bausch & Lomb colorimeter equipped with $\frac{1}{2}$ " test tubes.

Kinetic measurements. Calcium hydroxide (2.4 g.) and 100 ml. of distilled water were added to the reaction flask and heated to 45° C. When this temperature was attained, 100 ml. of cupric sulfate solution, preheated to 45° C., were added with agitation and the time of addition recorded. A 10-ml. sample was immediately withdrawn with a pipette and subsequent samples were taken at various intervals after mixing. The remaining material was stored in a Mason jar for seven months and a final sample taken when the crystallization process approached completion. During the initial reaction period the Bordeaux mixture was stirred at moderate speed and kept protected from the air.

Measurements of the amount of soluble copper were made by withdrawing a 10-ml. sample from the batch, adding it to 5 ml. of sucrose solution, and shaking gently at room temperature. After exactly four minutes *ca.* 5 ml. of the solution were filtered through a fritted glass disk under vacuum. A second filtration through the same disk was made immediately since the forerun usually came through cloudy. The optical density at $650\text{ m}\mu$ was then determined with the Bausch & Lomb colorimeter using distilled water as a blank.

The percentage conversion of the Bordeaux mixture from the amorphous to the crystalline state at any given time was then calculated from the relation

$$\text{per cent conversion} = 100 \frac{D_o - D}{D_o - D_f} \quad (1)$$

where D_o is the optical density obtained on the sample taken immediately after mixing, D_f is the optical density after seven months, and D the optical density obtained at any intermediate time. The results were then plotted on rectangular coordinates and the time required for 25 per cent conversion ($t_{1/4}$) estimated by interpolation. The induction period (t_i) was taken at the point where two lines projected from the lag period and the steep portion of the curve intersected.

EXPERIMENTAL RESULTS

The copper precipitate from 10-10-100 Bordeaux mixture was found to dissolve readily in 10 per cent aqueous sucrose solution. After filtration to remove undissolved calcium sulfate a deep blue solution containing chelated copper was obtained with an absorption maximum at $650 \text{ m}\mu$ (Fig. 1). The occurrence of a significant difference between the rates at which the precipitated copper compounds in fresh and aged Bordeaux were dissolved made possible the development of a method for determining

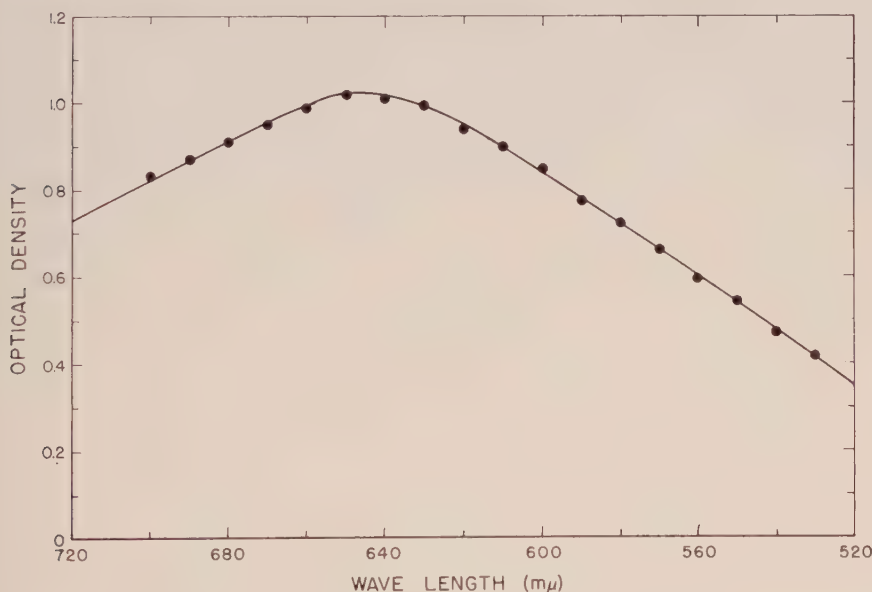


FIGURE 1. Absorption spectrum of copper-sucrose chelate at a concentration of 3.0 g./l. Cu^{++} .

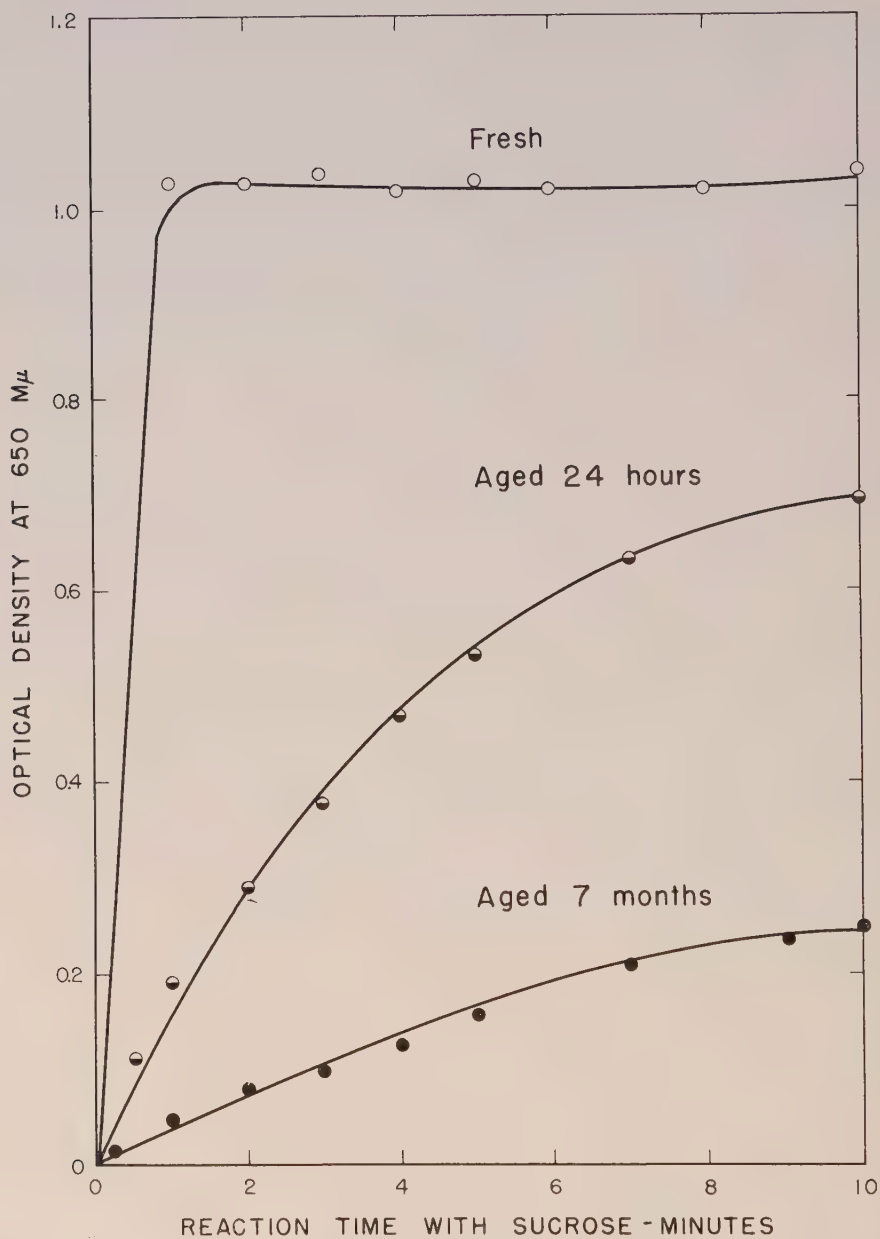


FIGURE 2. Rate of solubilization of freshly prepared and aged Bordeaux mixtures in sucrose.

the rate of conversion of the mixture from its original state as an amorphous hydrogel to its crystalline form. It was found that the copper constituents of fresh Bordeaux were almost completely chelated in four minutes, while mixtures which had been aged 24 hours and 7 months were chelated to the extent of 25 and 14 per cent respectively when shaken with sucrose solutions for the same length of time (Fig. 2). This corresponded to a maximum decrease of 0.86 in optical density which was large enough to permit accurate measurement of the crystallization rate.

Since almost all of the freshly prepared mixture dissolved in four minutes the reproducibility of measurement could be expected to approximate that of the colorimetric method of analysis, but on aged samples the amount of copper dissolved would be more critically dependent on time since measurements would be made in a region where the curve was still rising (Fig. 2). However, replicated measurements made on samples aged for 24 hours showed that the optical density readings could be reproduced to within 0.25 ± 0.006 . Although subsequent experiments showed that all of the copper in the freshly prepared mixture could be dissolved in one minute, retention of the four-minute interval was considered desirable to insure complete reaction.

In order to establish the usefulness of this method for determining crystallization rate, a 10-10-100 Bordeaux mixture was prepared at 45° C., and aliquots withdrawn at various time intervals and subjected to the chelation test with sucrose. When optical density values were plotted against time after mixing, a smooth sigmoid curve was obtained such as would be expected for a process of this type (Fig. 3). These values were then calculated as per cent conversion to the crystalloid by substitution in equation (1) making the assumption that crystallization would be substantially complete at the end of seven months and that the reading made on this sample would correspond to ca. 100 per cent conversion (Table I).

TABLE I
RATE OF CONVERSION OF BORDEAUX MIXTURE TO THE CRYSTALLINE STATE AT 45° C.

| Time after mixing (minutes) | Optical density at 650 m μ | Per cent conversion |
|--------------------------------|-----------------------------------|------------------------|
| 0 | 1.03 | 0 |
| 17 | 1.02 | 1.3 |
| 21 | 1.01 | 2.5 |
| 24 | 0.94 | 12 |
| 27 | 0.78 | 32 |
| 30 | 0.66 | 48 |
| 35 | 0.54 | 62 |
| 40 | 0.52 | 65 |
| 55 | 0.49 | 68 |
| 72 | 0.45 | 73 |

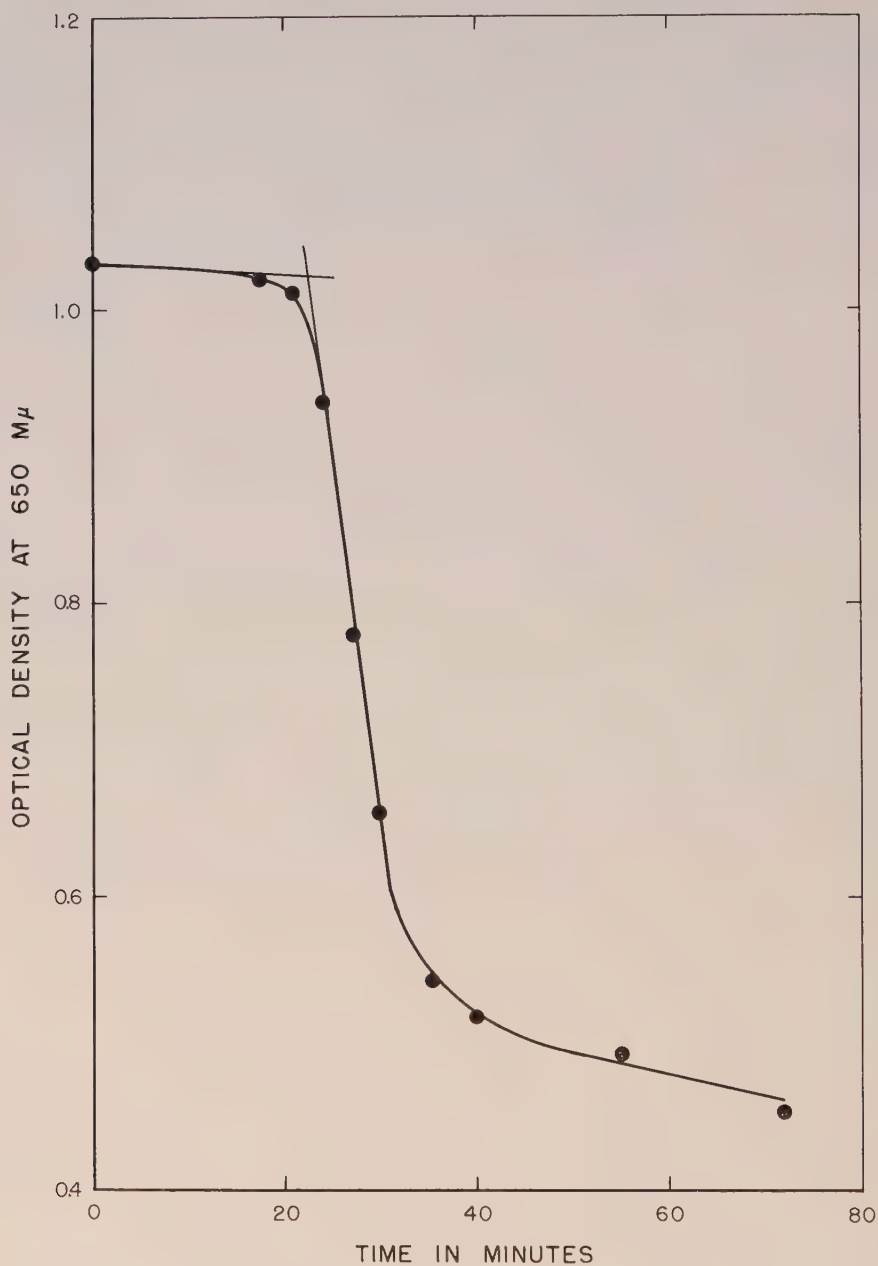


FIGURE 3. Change in optical density of filtered test solutions during aging of 10-10-100 Bordeaux mixture at 45° C.

TABLE II

COMPARISON OF PARAMETERS FOR 10-10-100 BORDEAUX DETERIORATION CURVES
RUN AT 45° C. IN PRECISION EXPERIMENTS

| Quarter-life ($t_{1/4}$) | Half-life ($t_{1/2}$) | Induction period (t_i) | Slope (m) |
|-------------------------------|----------------------------|-------------------------------|------------------|
| 27 | 34 | 23 | 5.2 |
| 29 | 34 | 25 | 5.2 |
| 27 | 34 | 22 | 5.5 |
| 27 | 36 | 23 | 5.2 |
| 27 | 34 | 22 | 5.8 |
| 28 | 34 | 23 | 6.5 |
| 28 | 36 | 23 | 5.6 |
| Av. 27.6 | 34.6 | 23.0 | 5.6 |
| σ 2.00 | 2.45 | 2.45 | 1.15 |

After 55 minutes the conversion was 68 per cent, so even though the reaction was still continuing slowly at the end of this time it was evident that the most profound changes had taken place within the first hour.

During the early part of this work deterioration was expressed in terms of half-life ($t_{1/2}$) since this quantity is generally used in kinetic measurements. In a series of experiments run under carefully controlled conditions it was found that the half-life could be satisfactorily reproduced (Table II). However, under routine conditions the induction period (t_i) and quarter-life ($t_{1/4}$) were less sensitive to experimental variations and could be determined more accurately (Table III). This was apparently due to the fact that the period of rapid change was often near completion at 50 per cent conversion to the crystalloid and measurements might have been made in a region where the slope of the curve was changing. The slope of the steep portion of the curve (m) was also found to be a useful parameter (Table III), but it could not be determined as accurately as the induction period or the quarter-life.

TABLE III

COMPARISON OF PARAMETERS FOR 10-10-100 BORDEAUX DETERIORATION CURVES
RUN AT 45° C. IN ROUTINE EXPERIMENTS

| Quarter-life ($t_{1/4}$) | Half-life ($t_{1/2}$) | Induction period (t_i) |
|-------------------------------|----------------------------|-------------------------------|
| 32 | 40 | 26 |
| 26 | 37 | 19 |
| 27 | 41 | 22 |
| 29 | 41 | 23 |
| 29 | 40 | 26 |
| 28 | 47 | 25 |
| 25 | 57 | 19 |
| 26 | 37 | 23 |
| Av. 28.0 | 42.5 | 22.9 |
| σ 6.00 | 17.50 | 7.42 |

If the experiments were to be confined to describing the behavior of 10-10-100 Bordeaux mixture at 45° C. it would be preferable to express the results in terms of induction period since this quantity is independent of a knowledge of the final state of the mixture. However, in more dilute mixtures, crystallization begins immediately after mixing, so it is impossible to compare results in these terms. Under conditions where the rate of crystallization is extremely slow it is often not feasible to determine the final state of the product so the use of the induction period is necessary. In view of these conflicting requirements it seems best to retain both parameters and use one or the other as the situation demands.

DISCUSSION

Sucrose has been used for the stabilization of Bordeaux mixture for many years (2, 3, 10) but its significance as a chelating agent has not been clearly understood. Holland *et al.* (4) recognized its solubilizing action on copper in dialysis experiments and proposed the formation of copper gluconate through a hydrolysis and oxidation process. Hockenyos (3) used it as a preservative and noticed that the precipitate and supernatant took on a deep blue color when it was added. McCallan and Wilcoxon (7) noted that neutral sodium malate will dissolve large amounts of copper from Bordeaux mixture so evidently this material acts as a chelating agent as well. Lieser and Ebert (5) prepared a sucrose-copper complex by dissolving the sugar in 3.2 *N* tetraethylammonium hydroxide and digesting the mixture with cupric hydroxide. Ammonium hydroxide saturated with $\text{Cu}(\text{OH})_2$ was then added in the cold and the complex precipitated with methanol. They give the empirical formula as $\text{C}_{12}\text{H}_{24}\text{O}_{15}\text{Cu}_3$.

The complex used in this work was obtained as a deep blue solution having a pH of 12. A precipitate was formed on the addition of acid which was redissolved by alkali. In the analysis of neutral or acid Bordeaux mixtures it is necessary to add sodium or calcium hydroxide to the reagent.

The usefulness of velocity of solubilization based on chelation as a method for assessing the aging of Bordeaux mixture is probably dependent to a large extent upon physical factors. When freshly prepared, the material is a hydrated gel which will mix intimately with the sugar solution and provide a very large reaction surface. Once crystallization of the copper compound begins to take place, the surface available for reaction is probably reduced owing to the growth of particles which can be attacked only at their surfaces. Since these ultimately become quite large it is understandable that the rate of solubilization should decrease.

The relationship between amount of copper solubilized and extent of crystal growth is difficult to express in exact terms; nevertheless there seems to be a direct correlation between them. When the optical density of the filtered solution is plotted against time (Fig. 3) a skewed sigmoid

curve is obtained such as would be expected for a crystallization process of this kind. This can also be expressed in terms of percentage conversion to crystallinity by substituting values for optical density in equation (1) and plotting the conversion against time. The scale is arbitrary but the results are reproducible with satisfactory precision.

The initial induction period may be looked at as the time required for nuclei to form, around which crystals can grow. This is followed by a period of rapid growth which finally falls off as the supply of amorphous material becomes exhausted. The most significant point on this curve is the induction period since it is a measure of the useful life of 10-10-100 Bordeaux mixture prepared and aged under these conditions.

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EFFECT OF LIGHT ON THE CATALASE AND CYTOCHROME OXIDASE ACTIVITIES OF LEAF TISSUES OF GREEN AND ALBINO SUNFLOWER PLANTS¹

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SUMMARY

Green and albino sunflower plants were grown in solution culture and the plants were exposed to various periods of continuous darkness and to prevailing seasonal diurnal light-dark conditions. Catalase and cytochrome oxidase activities of leaf tissues from both treatments were determined at intervals. Data show that both green and albino tissues subjected to continuous darkness exhibited higher catalase and cytochrome oxidase activities than did tissues subjected to diurnal light-dark conditions. Activities of both enzymes increased with length of time plants were exposed to continuous darkness. The relative increase in catalase activity of albino plants in the dark over those in diurnal light-dark conditions was greater than that of green plants. This was not the case for cytochrome oxidase activity.

INTRODUCTION

During the course of investigations on the effect of different iron and manganese nutrient levels on the activity of iron-containing enzymes of green and albino sunflower leaves (19), considerable daily variations in catalase activity were noted during the months of December and January. A comparison of catalase activities with light intensity \times time values recorded from a pyrliometer revealed that catalase activities of green leaf tissues were correlated with these values for the day preceding enzyme activity determinations. Since all leaf tissues for enzyme activity determinations were harvested at 8:30 A.M., plants were subjected to the usual period of from 14 to 16 hours of darkness and to from 1 to 2 hours of early morning light just prior to harvesting. Therefore, the period during which prolonged light conditions would be most likely to affect enzyme activity was during the daylight interval of the previous day. When catalase activities were compared with light intensity \times time values of the previous day, it was found that high catalase activity corresponded to light of relatively low intensity, and low catalase activity to light of relatively high intensity.

An experiment was designed to study the effects of prolonged dark and of diurnal light-dark treatments on the catalase and cytochrome

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oxidase activities of leaf tissues from green and albino scions grafted to green sunflower plants.

MATERIALS AND METHODS

Seeds of Russian sunflower (*Helianthus annuus* L.) yielding about 8 per cent albino seedlings⁴ were sown in flats of washed quartz sand on January 26, 1953. After emergence, the albino seedlings were discarded. On February 5, 16 solution cultures were set up, each consisting of a one-gallon, wide-mouthed glass jar containing 3 sunflower seedlings. All cultures received the following nutrient solution: macronutrient salts—0.001 *M* KH_2PO_4 , 0.005 *M* $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.002 *M* $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.002 *M* K_2SO_4 ; micronutrient elements—0.50 p.p.m. Fe, 0.25 p.p.m. Mn, 0.10 p.p.m. Zn, 0.10 p.p.m. B, 0.01 p.p.m. Cu, and 0.01 p.p.m. Mo.

Nutrient salts used were of analytical grade and nutrient solutions were prepared with single-distilled water. Solutions were supplied by the continuous renewal method (10, 11).

At 8:30 A.M. on March 9, 8 cultures of green plants were placed in the dark in a tent made from black muslin. White cotton sheets were placed over the tent to reduce heat absorption. A fan was placed at one end in such a position as to draw air through the tent, to provide within the tent a temperature and relative humidity as nearly equal as possible to that in the greenhouse. The remaining 8 cultures were subjected to prevailing diurnal light-dark conditions.

Terminal leaf tissues from each plant were removed for catalase determinations after periods of 6, 24, and 48 hours of dark or diurnal light-dark treatment, and for cytochrome oxidase determinations after 24 and 48 hours.

A second series of plants was grown at a later date for the determination of catalase and cytochrome oxidase activities of green and albino sunflower leaf tissues. On March 20, 1953, 200 sunflower seeds were sown in washed quartz sand. On April 10, 80 green seedlings were transplanted into 4-inch clay pots of washed quartz sand. On April 20, 300 seeds yielding about 20 per cent albino seedlings were planted as before. Upon emergence, green and albino seedlings were cleft-grafted to the green stock plants (16, 17, 18, 19). A total of 33 albino and 30 green seedlings were grafted. Only 6 albino and 13 green grafts united successfully. On May 20, 6 solution cultures were set up, each culture containing 1 albino and 2 green grafted plants. Plants in these cultures received the same nutrient treatment as described earlier.

At 8:30 A.M. on May 25, 2 cultures were placed in the tent under conditions similar to those described above, and the remaining cultures were exposed to diurnal light-dark conditions.

⁴ Seeds were provided through the courtesy of Dr. Raymond H. Wallace, Department of Botany, University of Connecticut, Storrs, Connecticut.

After 72 hours of dark or diurnal light-dark treatment, green and albino leaf tissues from each plant were removed for determinations of catalase and cytochrome oxidase activities.

Catalase activity was determined by measuring manometrically the amount of oxygen evolved from hydrogen peroxide (12, 19) and activity is expressed as milliliters of oxygen evolved per minute per milligram of protein nitrogen. Cytochrome oxidase activity was determined by measuring the rate of oxidation of ferrocytochrome c spectrophotometrically (15, 18, 19, 20) and activity is expressed as the first-order reaction constant. Each determination was run in triplicate and repeated once or twice on separate tissue aliquots and the data presented represent averages of these replications. Protein nitrogen content of the tissues used was determined by extracting small samples of freeze-dried leaf tissues for 16 hours with a refluxing water-alcohol mixture (14). Extracted tissue was then wet-digested and the total insoluble nitrogen content determined by the semi-micro Kjeldahl procedure. Enzyme activity is expressed on the basis of protein nitrogen present in the leaf tissues.

RESULTS

Catalase and cytochrome oxidase activities of green and albino leaf tissues subjected to the different light or dark treatments are shown in Tables I and II.

The data show that green and albino tissues of plants exposed to continuous darkness exhibited higher catalase and cytochrome oxidase activities than did similar tissues of plants exposed to diurnal light-dark conditions. In the first experiment activity of both enzymes in green tissues increased with length of time plants were exposed to continuous darkness, while activities showed small indefinite variations in plants sub-

TABLE I

CATALASE ACTIVITY FOR REPLICATE DETERMINATIONS OF GREEN AND ALBINO SUNFLOWER LEAF TISSUES OF PLANTS SUBJECTED TO DIFFERENT PERIODS OF CONTINUOUS DARKNESS OR DIURNAL LIGHT-DARK CONDITIONS. ACTIVITY IS EXPRESSED AS ML. O₂ EVOLVED/MIN. X MG. PROTEIN NITROGEN

| Time from start of experiment at 8:30 A.M., hours | Diurnal light-dark conditions | | | Continuous darkness | | |
|--|-------------------------------|------|------|---------------------|------|------|
| | Green leaf tissues | | | | | |
| 6 | 12.9 | 13.3 | 12.5 | 16.8 | 17.4 | 16.4 |
| 24 | 11.4 | 10.5 | 10.7 | 20.8 | 19.8 | 18.9 |
| 48 | 13.3 | 13.0 | 12.8 | 27.3 | 26.2 | 26.6 |
| 72* | 10.9 | 10.6 | — | 17.2 | 17.9 | — |
| | Albino leaf tissues | | | | | |
| 72* | 4.5 | 4.2 | — | 13.5 | 13.9 | — |

* Determinations made on leaf tissues from grafted sunflower plants grown almost 3 months later than those for which other catalase activity values are shown.

TABLE II

CYTOCHROME OXIDASE ACTIVITY FOR REPLICATE DETERMINATIONS OF GREEN AND ALBINO SUNFLOWER LEAF TISSUES OF PLANTS SUBJECTED TO DIFFERENT PERIODS OF CONTINUOUS DARKNESS OR DIURNAL LIGHT-DARK CONDITIONS. ACTIVITY IS EXPRESSED AS K SEC.⁻¹; MG. PROTEIN NITROGEN IN 3 ML.

| Time from start of experiment at 8:30 A.M., hours | Diurnal light-dark conditions | | Continuous darkness | |
|---|-------------------------------|-------|---------------------|-------|
| | Green leaf tissues | | | |
| 24 | 0.361 | 0.370 | 0.519 | 0.461 |
| 48 | 0.323 | 0.309 | 0.615 | 0.594 |
| 72* | 0.394 | 0.387 | 0.490 | 0.494 |
| | Albino leaf tissues | | | |
| 72* | 0.232 | 0.222 | 0.268 | 0.271 |

* Determinations made on leaf tissues from grafted sunflower plants grown almost 3 months later than those for which other cytochrome oxidase activity values are shown

jected to diurnal light-dark conditions. Green leaf tissues from grafted plants grown about 3 months later and subjected to 72 hours of continuous darkness or to diurnal light-dark treatments did not show such large differences in catalase and cytochrome oxidase activities as in the first experiment, although albino tissues showed a striking increase in catalase activity after this dark treatment. Green leaf tissues from grafted plants grown under diurnal light-dark conditions exhibited a catalase activity 2.5 times greater than did albino tissues from plants grown under the same conditions (Table I). However, green leaf tissues from plants subjected to 72 hours of darkness had a catalase activity only 1.3 times greater than did albino tissues from the same treatment. Thus the relative difference in catalase activity between that of albino plants in the dark and those under diurnal light-dark conditions was greater than that of green plants. In the case of cytochrome oxidase, the relative difference in activity between that of green plants in the dark and those in light-dark conditions was greater than that of albino plants (Table II). Enzyme activity has been expressed on a protein nitrogen basis, and it should be pointed out that gross changes in protein nitrogen content were small; therefore, it is believed that they did not significantly affect the data presented.

DISCUSSION

Results of this experiment indicate that daily alterations in the catalase activity of green sunflower leaf tissues which were previously noted may have been caused by variations in prevailing light intensity inasmuch as prolonged periods of darkness increased catalase activity. It seems apparent that long artificially-induced dark periods result in marked alterations within the leaf tissues which are favorable to an increased activity of catalase. The stimulation of cytochrome oxidase activity by dark treat-

ment serves to emphasize the likelihood that plant respiratory patterns are influenced by changes in light intensity. Metabolic activities within the leaf which result in a higher proportion of iron available for incorporation into enzymes may account for increased enzyme activity.

Ingalls and Shive (6) have reported that under conditions of high light intensity, internal changes take place within the plant which render iron less available, while under conditions of low light intensity iron becomes more available. Unpublished data (18) on experiments with Havana Seed tobacco demonstrated that prolonged dark treatment increased the water-soluble iron content of leaf tissues when compared with leaf tissues of plants subjected to diurnal light-dark treatment. It is believed that this soluble iron fraction represents a reservoir of iron which may be drawn upon for metabolic use including enzyme synthesis. However, an increased pool of available iron *per se* in the dark cannot account for an increased iron enzyme activity. In addition to available iron there must be a sufficient amount of suitable protein for apoenzyme synthesis as well as of porphyrin for heme synthesis. Therefore, dark-induced alterations within the tissues must represent either a continuing synthesis or an increasing availability of existing porphyrin-containing compounds, such as protochlorophyll (3, 9, 13) and chlorophyll. Available apoenzyme may be supplied by increased hydrolysis of existing protein moieties and/or by an increased synthesis of proteins of this type. Perhaps such shifts in protein metabolism take place at the expense of existing enzyme systems.

The greater effect of dark treatment on the catalase activity of albino than of green tissues suggests that the albino is capable of synthesizing the porphyrin nucleus. Since no chlorophyll is synthesized by the albino plants, we may postulate that porphyrin synthesis in these tissues advances to the state of formation of protoporphyrin IX or even protochlorophyll (4, 5), further steps toward chlorophyll production being blocked by genetic alterations in these tissues.

It seems apparent that destruction of catalase is not necessarily related to oxidative mechanisms associated with photosynthesis or to photooxidation mediated by chlorophyll since response of albino leaves to the different light and dark treatments is more marked, at least in the case of catalase activity, than is the response of green leaves.

Eyster (2) and Appleman (1) have reported effects of light on catalase activity in etiolated green corn seedlings and in barley seedlings, respectively. Eyster concluded that the decrease in catalase activity of etiolated seedlings after exposure to light was due to photooxidation of the enzyme, a concept with which Appleman (1) does not concur. Lavorel (7, 8) has reported that catalase activity of chloroplast suspensions decreases in light for 1 to 2 minutes, at which time it is light-saturated and reaches equilibrium. If placed in darkness, activity goes back to its original value in about the same time. He rejects the hypothesis that this is a simple

photochemical destruction of catalase.

From the experiments reported here, it is concluded that the interpretation of catalase or cytochrome oxidase activity data may be complicated by daily variations in light intensity, especially when determinations are made from one series of plants over a period of several days.

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SUPPRESSION OF TOMATO PLANT TERMINAL GROWTH BY α -CYANOCINNAMIC ACIDS AND RELATED COMPOUNDS¹

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SUMMARY

α -Cyano-2,4-dihalocinnamic acids, when applied as foliage sprays to seedling tomato plants, caused inhibition of stem elongation and rosetting of new growth. In order to determine which modification of this molecule would induce maximum activity, this study was undertaken.

Activity of α -cyanocinnamic acids was found to be correlated with substitution on the benzene ring much the same as the phenoxyacetic acid series. The monosubstituted α -cyanocinnamic acids showed greatest growth inhibition when the substituent on the *para* position was either chlorine or bromine. Maximum activity with the disubstituted compounds was associated with a bromine or chlorine atom in the 2 and 4 position of the benzene ring.

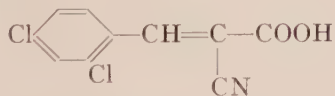
The apparent need for the cyano group on the α carbon atom was demonstrated. Maximum activity among the 2,4-dihalocinnamic acid derivatives was obtained only with those compounds which possessed this α -cyano group.

A possible function of the different parts of the α -cyano-2,4-dihalocinnamic acid molecule is discussed.

INTRODUCTION

α -Cyano-2,4-dichlorocinnamic acid described by Ligett *et al.* (7) in 1952 was shown to inhibit stem elongation and induce a rosette type of growth in young tomato plants. Since there is a need for developing a better understanding of the processes of plant growth suppression and stimulation, further studies were initiated in cooperation with Dr. W. B. Ligett and his colleagues at the Ethyl Corporation Research Laboratories. The primary purpose of the cooperative research was to determine the possible relationship of chemical structure to the biological properties, such as reduction of internodal distance, rosetting of new growth, and incomplete expansion of new leaves and to modify the molecule so as to enhance these types of activity as much as possible.

The basic structure of the ring halogenated α -cyanocinnamic acids has four points of primary interest: the carboxyl group, the benzene ring, the halogen substitutions on the benzene ring, and the α -cyano group. The double bond of the side chain is a possible point of reaction and is, therefore, of some interest. The molecular structure of α -cyano-2,4-dichlorocinnamic acid is as follows:



¹ The work reported upon in this paper was supported by Ethyl Corporation, 100 Park Avenue, New York, N. Y.

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In order to compare the effect changes on the basic structure had upon suppression of stem elongation, 49 compounds were synthesized. A brief summary of the data obtained when these compounds were sprayed on young tomato plants is presented.

MATERIALS AND METHODS

The following compounds, except compound No. 39, were prepared and their identities established by Dr. Ligett and his associates of the Ethyl Corporation Research Laboratories.

| No. | Chemical tested |
|-----|--|
| 1 | α -Cyano-2,4-dichlorocinnamic acid |
| 2 | α -Cyano-2,4-dichlorocinnamic acid sodium salt |
| 3 | α -Cyano-2,4-dichlorocinnamic acid diethanolamine salt |
| 4 | α -Cyano-2,4-dichlorocinnamic acid triethanolamine salt |
| 5 | α -Cyano-2,4-dichlorocinnamic acid phenylmercuric salt |
| 6 | Methyl α -cyano-2,4-dichlorocinnamate |
| 7 | Ethyl α -cyano-2,4-dichlorocinnamate |
| 8 | Butoxyethoxypropyl α -cyano-2,4-dichlorocinnamate |
| 9 | α -Cyano-2,4-dichlorocinnamanilide |
| 10 | Ethyl α -cyano- β -(trichloromethyl)acrylate |
| 11 | Ethyl α -cyano- β - <i>n</i> -hexyl- β -methylacrylate |
| 12 | Ethyl α -cyano- β -cyclohexyl- β -methylacrylate |
| 13 | Ethyl α -cyano- β -thenylacrylate |
| 14 | Ethyl α -cyano- β -(<i>o</i> -chloroanilino)acrylate |
| 15 | Ethyl α -cyano- β -(<i>p</i> -chloroanilino)acrylate |
| 16 | Ethyl α -cyano- β -(<i>p</i> -chlorophenyl)- <i>p</i> -chlorocinnamate |
| 17 | α -Cyanocinnamic acid |
| 18 | α -Cyano- <i>o</i> -chlorocinnamic acid |
| 19 | α -Cyano- <i>m</i> -chlorocinnamic acid |
| 20 | α -Cyano- <i>p</i> -chlorocinnamic acid |
| 21 | α -Cyano-3,4-dichlorocinnamic acid |
| 22 | α -Cyano-2-hydroxy-5-chlorocinnamic acid |
| 23 | α -Cyano-2-methoxy-5-chlorocinnamic acid |
| 24 | α -Cyano- <i>p</i> -bromocinnamic acid |
| 25 | α -Cyano-2-hydroxy-5-bromocinnamic acid |
| 26 | α -Cyano- <i>p</i> -hydroxycinnamic acid |
| 27 | α -Cyano-3-methoxy-4-hydroxycinnamic acid |
| 28 | α -Cyano- <i>p</i> -methoxycinnamic acid |
| 29 | α -Cyano-3,4-dimethoxycinnamic acid |
| 30 | α -Cyano- <i>p</i> -isopropylcinnamic acid |
| 31 | α -Cyano-3,4-dichlorocinnamic acid diethanolamine salt |
| 32 | Ethyl α -cyano-3,4-dichlorocinnamate |

- 33 Ethyl α -cyano-2,3,4,5,6-pentachlorocinnamate
- 34 Ethyl α -cyano-2,4-dibromocinnamate
- 35 Ethyl α -cyano-*p*-(2,4-dichlorobenzyloxy)cinnamate
- 36 2,4-Dichlorocinnamic acid
- 37 α -Methyl-2,4-dichlorocinnamic acid
- 38 α -Ethyl-2,4-dichlorocinnamic acid
- 39 α -Cyano-2,4-dichlorohydrocinnamic acid
- 40 α -Cyanobenzyl cinnamate
- 41 α -Cyano-*o*-methoxybenzyl cinnamate
- 42 α -Cyano-*p*-methoxybenzyl cinnamate
- 43 *N,N*-Diethylcinnamide
- 44 Cinnamic-2-methylpiperidide
- 45 *p*-Chlorocinnamic acid
- 46 β -Chloroethyl *p*-chlorocinnamate
- 47 β -Diethylaminoethyl *p*-chlorocinnamate
- 48 *p*-Chlorophenyl *p*-chlorocinnamate
- 49 Benzyl *p*-chlorothiocinnamate

α -Cyano-2,4-dichlorohydrocinnamic acid was prepared by Dr. E. A. Prill and W. R. Smith of Boyce Thompson Institute. The synthesis of this compound was through the reaction of α ,2,4-trichlorotoluene with the sodium derivative of ethyl cyanoacetate, and was similar to the method used by Walker (15) for the preparation of its unchlorinated analogue. The intermediary product (ethyl α -cyano-2,4-dichlorocinnamate) distilled at 143–144° C. at 3 mm. Upon hydrolysis this yielded the free acid which was recrystallized from methanol. This acid had a melting point of 150–151° C. and contained 5.69 per cent nitrogen as compared to 5.74 per cent calculated for the anticipated compound corresponding to the formula $C_{10}H_7O_2NCl_2$.

The compounds were soluble either in water or acetone. All compounds except the salts were dissolved in acetone at a ratio of 1:10 (weight/volume) in a large beaker. Triton X-155 emulsifier (Rohm & Haas) was added in an amount to make its concentration 0.1 per cent in the final suspension. Water was poured into the acetone solution in order to bring it to the desired volume. This produced a suspension of very small particles approaching colloidal dimensions for all compounds. The salts were dissolved in water containing 0.1 per cent of Triton X-155. Suspensions or solutions were then diluted in water to obtain the desired lower concentrations.

Each aqueous suspension or solution was then atomized onto three or four potted 25- to 28-day-old tomato (*Lycopersicon esculentum* Mill.) plants of the variety Bonny Best. The plants were placed on a rotating table in a spray hood, described and illustrated by McCallan (8), and were exposed to 100 ml. of material sprayed from a DeVilbiss paint touch-up

gun operating at an air pressure of 40 pounds for 30 seconds at a distance of 40 inches from the plants.

After drying, the tomato plants were returned to the greenhouse and maintained at an ambient temperature of 18° to 30° C. In each experiment 12 or more plants of comparable size and vigor were sprayed with an aqueous solution of emulsifier and acetone and held as untreated standards. All plants were measured from the cotyledonary node to the terminal bud prior to spraying and 3, 5, 7, and 14 days thereafter. Data are reported as percentage inhibition of stem elongation (growth) on the 14th day only since maximum effects were observed at this time.

EXPERIMENTAL RESULTS

Stem elongation above the cotyledonary node was reduced from 0 to 92 per cent by a concentration of 1024 p.p.m. of the various α -cyanocinnamic acids. The concentration of 1024 p.p.m. was chosen as the initial level to test all compounds because it was the maximum concentration of α -cyano-2,4-dichlorocinnamic acid that could be tolerated by the seedling tomato plants. Among the changes noticed on treated plants were shortened internodes with no reduction in the number of internodes. Free-hand sections of the pith tissue revealed cells about one-fourth the size of those found in untreated plants. From these observations it was concluded that the active α -cyanocinnamic acids operated by suppressing cell enlargement rather than cell division. This appeared to be true for all active compounds. The leaves on plants treated with the active compounds failed to enlarge and became abnormally firm and rigid. A trace of marginal necrosis was observed on some leaves which had been treated with 1024 p.p.m. of the α -cyano-2,4-dichlorocinnamic acid but this type of damage was of minor importance. Some plants, when treated with a concentration of 1024 p.p.m. of the growth inhibitor, were apparently weakened at the ground level and bent at that point. Adventitious roots developed where the stem touched the ground. Plants that showed the stem-collapse tendency were discarded. Tomato plants showing the typical response to α -cyano-2,4-dichlorocinnamic acid are shown in Figure 1 A.

The only other compound reported which suppressed stem elongation similarly to the α -cyanocinnamic acids under discussion was maleic hydrazide (5, 13). Figure 1 B shows the typical response of tomato plants sprayed with maleic hydrazide and with α -cyano-2,4-dichlorocinnamic acids. Maleic hydrazide, at concentrations higher than those reported here, causes stimulation of axillary shoot growth resulting in a bushy plant (13). In contrast, the α -cyanocinnamic acids produce a marked rosetting of new growth. The bluish coloration of the leaf veins observed on maleic hydrazide-treated plants was absent. Immature leaves, present at time of treatment, did not expand as much as those on plants treated with maleic

hydrazide. The growth suppression effect of the α -cyanocinnamic acids was apparent sooner but did not last as long as that induced by maleic hydrazide. Low concentrations of the α -cyanocinnamic acids markedly stimulated terminal elongation for a short period of time. This effect was



FIGURE 1. (A) The effect of α -cyano-2,4-dichlorocinnamic acid on tomato plants two weeks after spraying with 100 ml. of an aqueous solution of the compound. Left to right: control, sprayed with 512 p.p.m., sprayed with 1024 p.p.m. (B) The difference in appearance of tomato plants sprayed with 100 ml. of an aqueous solution. Left to right: control, 1024 p.p.m. maleic hydrazide, and 1024 p.p.m. α -cyano-2,4-dichlorocinnamic acid.

either absent or not pronounced with the concentrations of maleic hydrazide used in this work.

Effect of modifying the carboxyl group. Since the biological activity of 2,4-D has been modified by its esterification and conversion to various salts and amides, a series of such materials were tested. Data from two tests each on the inhibitory effects of nine derivatives of α -cyano-2,4-dichlorocinnamic acid are summarized in Table I.

The conversion of the acid to salts did not suppress the activity except

TABLE I

EFFECT OF SEVERAL ESTERS, SALTS, AND THE ANILIDE OF α -CYANO-2,4-DICHLOROCINNAMIC ACID IN SUPPRESSING STEM ELONGATION OF TOMATO PLANTS.
DATA PRESENTED ON AN ACID EQUIVALENT BASIS

| No. | Derivative tested | Suppression of stem elongation (%) at concn. of (p.p.m.) | | |
|-----|--------------------------|--|-----|-----|
| | | 1024 | 512 | 256 |
| 1 | Acid | 75 | 52 | 30 |
| 2 | Sodium salt | — | 34 | 12 |
| 3 | Diethanolamine salt | 73 | 56 | — |
| 4 | Triethanolamine salt | 63 | 57 | — |
| 5 | Phenylmercuric salt | * | * | * |
| 6 | Methyl ester | * | * | * |
| 7 | Ethyl ester | * | * | 69 |
| 8 | Butoxyethoxypropyl ester | * | 80 | 60 |
| 9 | Anilide | 13 | — | — |

* Plants killed.

when the sodium salt was used. The phenylmercuric salt of α -cyano-2,4-dichlorocinnamic acid was so phytotoxic that the plants were killed within a week after treatment so no data on suppression of terminal growth are presented.

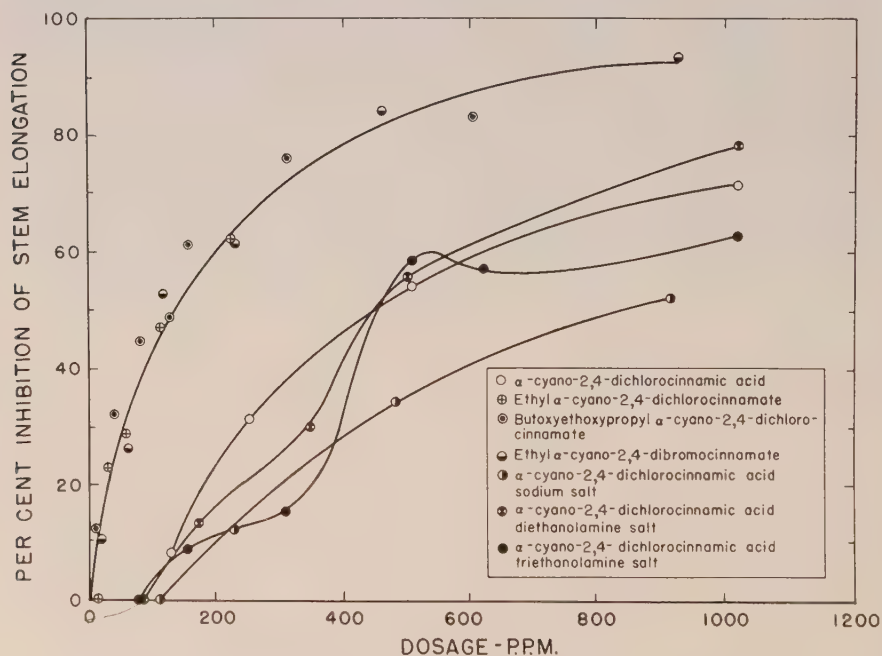


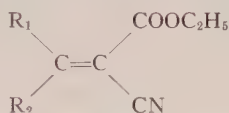
FIGURE 2. The effect of varying concentrations of different α -cyano-2,4-dihalocinnamic acids, esters, and salts on stem elongation of tomato plants when applied as a foliage spray. Data of esters and salts are based on acid equivalent.

The esters were definitely more phytotoxic than the free acid and also were more inhibitory to stem elongation. Activity of the esters decreased as the length of the carbon chain was increased.

The analide was found to be definitely less active than all other materials in this series.

Data from several tests showing the relative effectiveness of salts and esters of the acid are presented in Figure 2. The data indicate that the salts retain an activity approximating the free acid but the esters are both more phytotoxic at higher concentrations and more active as suppressors of stem elongation at lower concentrations than the free acid. These series of tests also show that phytotoxicity among the various esters was reduced

TABLE II
EFFECT OF STEM ELONGATION ON TOMATO PLANTS OF β -SUBSTITUTED ETHYL
 α -CYANOACRYLATES OF THE FORMULA



| No. | Compound tested | | Suppression of stem elongation (%) at concn. of 1024 p.p.m. |
|-----|---|------------------------|---|
| | Substitution on the β carbon atom | | |
| | R ₁ | R ₂ | |
| I | 2,4-Dichlorophenyl | Hydrogen | 61 |
| 10 | Trichloromethyl | Hydrogen | 0 |
| 11 | <i>n</i> -Hexyl | Methyl | 6 |
| 12 | Cyclohexyl | Methyl | 0 |
| 13 | Thenyl | Hydrogen | 0 |
| 14 | <i>o</i> -Chloroanilino | Hydrogen | 0 |
| 15 | <i>p</i> -Chloroanilino | Hydrogen | 0 |
| 16 | <i>p</i> -Chlorophenyl | <i>p</i> -Chlorophenyl | 0 |

as the aliphatic chain length increased, and that the dibromo ester was not as phytotoxic as its dichloro analogue.

Effect of β -substituents on the α -cyanoacrylic acids. Substantial evidence was obtained that the 2,4-dichlorophenyl group was essential for regulant activity. A series of compounds were prepared and tested comprising various β -substituted ethyl α -cyanoacrylates. The data in Table II show that none of these compounds were sufficiently active to be of interest.

Effect of various substitutions on the benzene ring of α -cyanocinnamic acid. Since it was obvious that the halogenated benzene ring was essential for activity of this class of compounds, a study was made of the effect of different substituents on this structure. The relative effectiveness of substitutions on the benzene ring is shown by data in Table III.

The several substituents exerted an effect on the α -cyanocinnamic acids similar to that reported by Muir *et al.* (11), McNew and Hoffmann (9), and

Zimmerman and Hitchcock (16) for a series of substituted phenoxyacetic acids. Halogenation is essential for maximum activity. Chlorination or bromination in the *para* position increases activity more than monosubstitution elsewhere on the benzene ring. Maximum effects were observed from 2,4-dihalogen substituted compounds. Hydroxyl and methoxyl groups were the least effective substituents tested.

Effect of various substitutions on the α carbon atom of 2,4-dichlorocinnamic

TABLE III

EFFECT OF VARYING SUBSTITUENTS ON THE BENZENE RING OF α -CYANOCINNAMIC ACID ON STEM ELONGATION OF TOMATO PLANTS. DATA OF THE SALTS AND ESTERS ARE PRESENTED ON AN ACID EQUIVALENT BASIS

| Compound tested | | Type of compound | Suppression of stem elongation (%) at concn. of (p.p.m.) | | |
|-----------------|--|---------------------|--|------|------|
| No. | Substituents and positions on the benzene ring | | 1024 | 512 | 256 |
| 17 | None | Acid | 5 | — | — |
| 18 | <i>o</i> -Chloro | Acid | 0 | — | — |
| 19 | <i>m</i> -Chloro | Acid | 0 | — | — |
| 20 | <i>p</i> -Chloro | Acid | 29 | 0 | — |
| 1 | 2,4-Dichloro | Acid | 68 | 55 | 28 |
| 21 | 3,4-Dichloro | Acid | 0 | — | — |
| 22 | 2-Hydroxy and 5-chloro | Acid | 15 | 8 | — |
| 23 | 2-Methoxy and 5-chloro | Acid | 0 | — | — |
| 24 | <i>p</i> -Bromo | Acid | 72 | 62 | 59 |
| 25 | 2-Hydroxy and 5-bromo | Acid | 5 | — | — |
| 26 | <i>p</i> -Hydroxy | Acid | 0 | — | — |
| 27 | 3-Methoxy and 4-hydroxy | Acid | 0 | — | — |
| 28 | <i>p</i> -Methoxy | Acid | 18 | 0 | — |
| 29 | 3,4-Dimethoxy | Acid | 8 | — | — |
| 30 | <i>p</i> -Isopropyl | Acid | 13 | 8 | — |
| 3 | 2,4-Dichloro | Diethanolamine salt | 73 | 56 | — |
| 31 | 3,4-Dichloro | Diethanolamine salt | 13 | 7 | 0 |
| 7 | 2,4-Dichloro | Ethyl ester | * | * | 69 |
| 32 | 3,4-Dichloro | Ethyl ester | 88 | 65 | — |
| 33 | Pentachloro | Ethyl ester | 25 | — | — |
| 34 | 2,4-Dibromo | Ethyl ester | 92** | 84** | 68** |
| 35 | <i>p</i> -(2,4-Dichlorobenzyloxy) | Ethyl ester | 0 | — | — |

* Plants killed.

** Concentrations of this compound used were 927, 463, and 232 p.p.m. respectively.

acid. The apparent need for the α -cyano group for maximum activity is demonstrated by the data in Table IV. The activity of α -cyano-2,4-dichlorocinnamic acid is lost if hydrogen, the methyl or the ethyl group replaces the cyano group. The apparent need for the α -cyano group is further demonstrated if we compare α -cyano-*p*-chlorocinnamic acid (No. 20) with *p*-chlorocinnamic acid (No. 45). The former compound suppressed terminal growth 29 per cent while the latter only suppressed growth 6 per cent.

Effect of the saturated and unsaturated bond between the α and β carbon atoms. The unsaturated bond between the α and β carbon atoms of α -

TABLE IV

EFFECT OF VARYING SUBSTITUENTS ON THE α CARBON ATOM OF 2,4-DICHLOROCINNAMIC ACID ON STEM ELONGATION OF TOMATO PLANTS

| Compound tested | | Suppression of stem elongation (%) at concn. of (p.p.m.) | |
|-----------------|---|--|-----|
| No. | Substituent on the α carbon atom | 1024 | 512 |
| 1 | Cyano | 68 | 50 |
| 36 | None | 0 | — |
| 37 | Methyl | 0 | — |
| 38 | Ethyl | * | 0 |

* Plants killed.

cyano-2,4-dichlorocinnamic acid allows the formation of *cis* and *trans* isomers. Both isomers have been synthesized, tested, and found to possess equal growth inhibitory activity.

Although all of the active growth inhibitors of this α -cyanocinnamic acid series tested have an unsaturated bond between the α and β carbon atoms, it is doubtful if this is necessary. The data presented in Table V show that the inhibitory characteristic of α -cyano-2,4-dichlorocinnamic acid is retained when the unsaturated bond is hydrogenated to form α -cyano-2,4-dichlorohydrocinnamic acid.

Effect of various cinnamic acid esters and amides. Although only the α -cyanocinnamic acids showed pronounced stunting effects on tomato plant stem elongation, it was of interest to test some cinnamic acid esters and amides. McNew and Hoffmann (9) have shown that ester and amide formation did not destroy biological activity in the phenoxyacetic acid series; therefore, it was reasonable to assume that any biological activity present in the cinnamic acid and *p*-chlorocinnamic acid would not be destroyed by ester or amide formation. The present tests show that none of the cinnamic acids or *p*-chlorocinnamic acid esters or amides (Nos. 40, 41, 42, 44, 45, 46, 47, 48, 49) were active except *N,N*-diethylcinnamide (No. 43).

TABLE V

EFFECT OF α -CYANO-2,4-DICHLOROCINNAMIC ACID AND α -CYANO-2,4-DICHLOROHYDROCINNAMIC ACID IN SUPPRESSING TOMATO PLANT STEM ELONGATION

| No. | Compound | Suppression of stem elongation (%) at concn. of (p.p.m.) | |
|-----|--|--|-----|
| | | 1024 | 512 |
| 1 | α -Cyano-2,4-dichlorocinnamic acid | 71 | 60 |
| 39 | α -Cyano-2,4-dichlorohydrocinnamic acid | * | 88 |

* Plants killed.

DISCUSSION

The most active growth inhibitors of the α -cyanodihalocinnamic acids possess a basic nucleus reminiscent of the phenoxyacetic acids. Both types of compounds possess a substituted benzene ring and a carboxyl group at the end of a short aliphatic chain. Many of the molecular changes associated with increased or decreased biological activity in the phenoxyacetic acids are found in the α -cyanocinnamic acids. The unsubstituted benzene ring in both series is not as active as the halosubstituted rings. Monosubstitution in the *para* position enhances the activity, while substitution in the *ortho* position reduces activity (11, 16). Veldstra (14) in his summary article stated that *m*-chlorophenoxyacetic acid will produce biological activity paralleling that found when the chlorine is in the *para* position. The α -cyanocinnamic acids do not follow this pattern. The greatest biological activity among the monochlorinated compounds is associated with the *para* position on the benzene ring.

Activity of α -cyanocinnamic acid with disubstitution on the benzene ring has similarities among the phenoxyacetic acids. Although biological activity is present when the α -cyanocinnamic acids are substituted in the 3 and 4 positions (4), the greatest activity is associated with the 2 and 4 positions. The reduced activity with chlorine in the 3 and 4 positions is similar to the results found by Leaper and Bishop (6) for substituted phenoxyacetic acids.

One marked difference in biological activity between the α -cyanocinnamic acids and the phenoxyacetic acids is the effect of substituting bromine in the 2 and 4 positions of the benzene ring. It has been reported (9) that the 2,4-dibromophenoxyacetic acid possesses biological activity corresponding to its 2,4-dichloro analogue. Data in Table III show that substitution of bromine for chlorine increases the activity. Ethyl α -cyano-2,4-dibromocinnamate is approximately one-fourth as toxic to tomato plants yet is as active as its chloro analogue at low concentrations. This same trend of increased biological activity holds true if we compare the *p*-bromo and *p*-chloro α -cyanocinnamic acids.

Although the function of the aryl group is not known, an hypothesis for it can be advanced. Muir and Hansch (10) postulated that the growth regulating compounds of the phenoxy and benzoic acid type and perhaps others are attached to a protein molecule by two points, the carboxyl group and a point on the aryl segment of the molecule. It is doubtful if the α -cyanocinnamic acid type of growth inhibitor reacts in such a manner. α -Cyano-2,4-dichlorocinnamic acid growth inhibitor can exist in a *cis* and *trans* form. The fact that both isomers possess equal biological activity brings one to the conclusion that the aryl group does not enter into a point of attachment. If the aryl groups were necessary for a point of attachment, it seems logical to surmise that only one isomer would pos-

sess activity as in the case of *cis*-cinnamic acid (14). From what is known of fungus toxicology, we may speculate that the halogenated aryl group acts as an aid in penetrating the lipoid layers of the cells. Rich and Horsfall (12) showed that the addition of a phenyl group increased the oil solubility of the 4-nitrosopyrazole group and that the increase in oil solubility was directly correlated with an increase in fungitoxicity.

Veldstra (14), in his discussion of the effect of substitution on the aliphatic chain of the phenoxyacetic acids, points out that the substitution of a methyl group on the α carbon atom has little effect on activity while substitution of two methyl groups reduces activity. The 2,4-dichlorocinnamic acids do not follow this pattern. The unsubstituted, as well as the compound where an α hydrogen atom was substituted with an ethyl or methyl group, were inactive. The only substitution on the α carbon atom found to impart activity to the molecule was the cyano group.

Among the cinnamic acid esters tested were many in which the cyano group was present on the alcohol part of the molecule instead of being associated directly with the α carbon atom of the cinnamic acid. None of these esters were effective growth inhibitors. This would be added confirmation that the cyano group on the α carbon atom is necessary for maximum growth inhibition.

Although the function of the cyano group is unknown, we can speculate as to the action it might perform. The fact that a large number of small cells are produced in plants treated with the growth inhibitor would indicate that cell division is progressing but enlargement is blocked. Baldovinos (1), Blank and Frey-Wyssling (2), Chao (3), and others have shown that the enlarging cell synthesizes nitrogenous compounds. If the synthesis of the nitrogenous compound were slowed down by some means, the cells would not enlarge. The α -cyanocinnamic acids may function in such a manner.

Although cinnamic acid esters and *p*-chlorocinnamic acid esters were tested, only one, *N,N*-diethylcinnamide, was active. Another amide-type compound, cinnamic-2-methylpiperidide, was inactive. McNew and Hoffmann (9) reported the formation of 2,4-D esters and amides did not destroy the biological activity; therefore, if the cinnamic acids were active, their esters and amides should be also. The fact that only one amide of the cinnamic acids is active would suggest that there may be a problem of *cis* and *trans* isomers or a problem of penetration through the lipoidal layer to the active site. It may be that during the formation of the amide, a preponderance of one isomer is formed. Veldstra (14) reports that *cis*-cinnamic acid is the biologically active form.

All the details of relationship between chemical structure and activity have not been worked out but enough has been done to outline the essential features necessary for activity in this group of compounds. The evidence

suggests that the esters of α -cyano-2,4-dihalocinnamic acids are the most active members of this series. The esters possess a biologically active nucleus and enough lipoid and water solubility to enter the plant and act as growth inhibitors.

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INDUCED POLYPLOIDY IN HORTICULTURAL VARIETIES OF VERBENA

CLYDE CHANDLER

SUMMARY

Tetraploidy was induced in the *Verbena hybrida* Cultivar Spectrum Red, by soaking seeds in aqueous solutions of colchicine. Tetraploidy was also induced in three other *Verbena hybrida* cultivars by treating growing points with an emulsion of colchicine. Triploids were obtained by crossing tetraploids with diploids. All polyploids failed completely in the production of seed after controlled self-pollinations were made in the greenhouse and in the field. This failure was due in part to a high percentage of aborted pollen and to the slow rate of pollen-tube growth. All diploid plants were highly self-fertile.

Polyploids exhibited the usual characteristics typical of induced polyploids. Increased vegetative vigor in leaves, flowers, and stems made the polyploids very desirable plants for bedding or for use as cut flowers. Five clones have been selected for introduction to the Horticultural Trade.

INTRODUCTION

Many horticultural plants have been improved by the induction of polyploidy through the use of colchicine. Among those introduced commercially are tetraploid snapdragons, forsythia, marigolds, phlox and gaillardia (7, 8, 13, 18). Tetraploids have also been produced in *Rudbeckia*, *Lobelia*, *Heimerocallis*, *Lilium longiflorum*, *Saintpaulia*, *Cosmos*, stock, wall-flower, *Salvia*, carnations, poinsettias, *Calendula* and chrysanthemums (1, 2, 3, 5, 9, 11, 14, 15, 16, 19, 20). No attempt has been made here to include all references on induced polyploidy in flowering plants. However, those mentioned above illustrate that through increase in flower size, shortening and thickening of flower scapes, and marked vegetative vigor, many have been improved by the induction of ploidy.

Verbenas were popular garden plants in the latter half of the 19th Century. In the list of plants certified from 1859 to 1893 by the Royal Horticultural Society, more than 100 first class certificates were recorded as awarded to garden varieties of verbenas. In 1868 a well-known seed company listed over 50 named varieties (17). During more recent years their popularity has decreased somewhat due in part to their undesirable habit of vegetative growth. The present experiments were initiated to determine the effect of the induction of polyploidy on vegetative and floral development.

In 1940 Furusato (6) listed *Verbena hybrida* among polyploid plants produced by colchicine. In 1949 Saito (14) figured a verbenas plant with diploid, tetraploid, and pentaploid branches produced from colchicine

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treatment. No further report on the value of these polyploids to horticulture has been located.

MATERIALS AND METHODS

For the present study which was begun in 1949, Cultivar Spectrum Red, a member of the grandiflora group of *Verbena hybrida* Voss was selected for colchicine treatment. Since tetraploids of this variety proved to be of considerable interest to horticulturists, polyploidy was induced in three other plants selected from a bed of mixed hybrid grandiflora verbena.

Induction of polyploidy. Seeds of Cultivar Spectrum Red were obtained from W. Atlee Burpee Co., Philadelphia, Pa. in 1949. They were soaked in 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 per cent aqueous solutions of colchicine for 2, 12, 16, 20, and 24 hours. A one-hour soaking period was also used for the four highest concentrations. Controls consisted of dry seeds and seeds soaked in tap water for 24 hours. One hundred seeds were used for each lot. After soaking, all seeds were planted in soil in the greenhouse.

Three plants were selected from a display bed of hybrid verbena growing at Yonkers, New York and were propagated vegetatively. When the cuttings were well established they were treated three times at intervals of three days by dropping 1.0 per cent colchicine emulsion on the growing points. As soon as the treated shoots showed any characteristics of polyploid tissue they were removed, rooted in sand, and later potted in good garden soil. All plants were grown to the flowering stage.

Identification of tetraploids. The size of pollen grains for diploid and polyploid plants was determined by placing the pollen on a glass slide and adding a drop of aceto-carmin stain. A cover glass completed the mount. The preparation was examined for uniformity in size of pollen and for the percentage of abortion. The diameter of ten grains in ten fields was recorded in ocular units. For these measurements large grains with cytoplasm were selected. In each field the total number of viable and aborted grains was recorded from which the percentage of abortion was calculated.

The chromosome numbers were determined from aceto-carmin smear preparations of pollen mother cells and acetic-orcein smears of root tips.

Fertility studies. All self-pollinations were fully controlled by complete isolation of individual plants or by placing the entire plant under a cheese-cloth-covered frame. Pollinations were made on plants growing in the greenhouse and in the field. Pollen from the same flower or from another flower of the same plant was placed on the stigma when it first appeared to be moist. In case of cross-pollinations flowers were carefully emasculated just prior to anther dehiscence and pollinated when the stigmas appeared to be receptive.

For testing the viability of the pollen, three drops of media made of 2 per cent agar with either 10, 15 or 20 per cent sucrose were placed on a

glass slide. As soon as the agar solidified, pollen was scattered over the surface of each drop. The slides were then placed in a moist chamber. After 24 hours the preparations were examined microscopically and the percentage of germination was determined.

One diploid and two triploid clones were selected for studies on the rate of pollen-tube growth. The diploid clone of Spectrum Red which is highly self-fertile was used. One triploid clone was obtained by crossing the tetraploid Spectrum Red with the diploid and the other triploid clone was produced by crossing a purple tetraploid with a Spectrum Red diploid. Both triploid clones failed to produce seed to fully controlled self- or close-pollinations. Plants in good bloom were selected and isolated. All fully opened flowers were removed. Fifty large buds were emasculated and left until the following morning when corollas were fully expanded and the stigmatic surfaces appeared moist and receptive. Pollen was collected from other plants of the same clone and used for pollination of the 50 previously emasculated flowers. Three pistils were collected from each of the three clones at intervals of two hours for the following 24 hours. Additional collections at 30 and 48 hours were made for the sterile triploids. The pistils were placed in vials containing a killing solution made by adding 7 ml. of commercial formalin (37 per cent) to 100 ml. of 70 per cent alcohol. Pistils were held in the fixative for several days after which they were removed, placed on a glass slide, carefully slit longitudinally with fine needles, and each side of the pistil flattened somewhat. A drop of acetocarmine stain applied to the cut surface and a cover glass completed the mount (4). Pollen tubes as they grew through the style toward the ovary were easily located by the density of the cytoplasm near the tip of the tube. Tips of the longest tubes were located in the style and marked by placing a red line on the lower surface of the slide. The entire length of the style in millimeters and the length of the longest tube were recorded. When studies were completed the rate of tube growth in the various preparations was calculated as percentage of stylar length.

RESULTS

Induction of polyploidy. Approximately 1,600 seedlings were grown from seeds of diploid Spectrum Red which were soaked in aqueous solutions of colchicine. The following treatments were most effective for the induction of tetraploidy: 0.5 per cent colchicine for 20 hours, 1.0 per cent colchicine for 16 and 20 hours, and 2.0 per cent colchicine for 12 and 16 hours.

As these seedlings flowered pollen grains were measured. Pollen from 40 diploid (control) plants measured 37.5 to 66.5 microns in diameter (Fig. 1 A). Pollen from 28 of these plants was uniform in size with 37.5 to 66.5 microns as a standard measurement of pollen from diploid plants. Sixty-nine of the seedlings from treated seed of Spectrum Red were con-

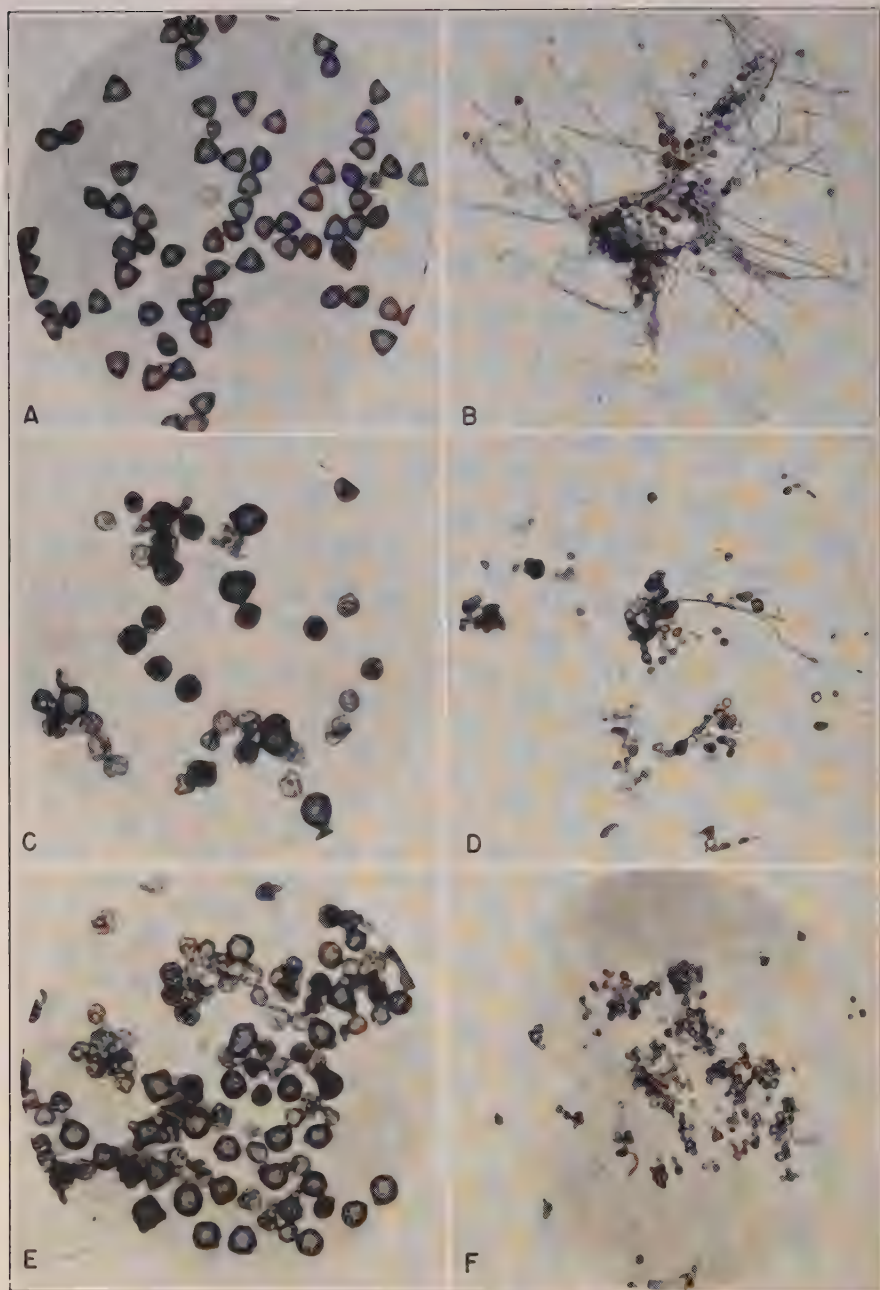


FIGURE 1. Pollen and pollen germination (A, B, diploid; C, D, tetraploid; E, F, triploid. pollen A, C, E ($\times 77$), germinating pollen B, D, F ($\times 27$).

sidered tetraploid since pollen from these plants was 37.5 to 95.0 microns with the majority of the viable grains 66.5 to 85.5 microns in diameter. Tetraploids obtained by colchicine treatment of the growing points of three selected plants also had pollen which measured 66.5 to 95.0 microns. Pollen from triploid plants obtained by crossing tetraploids with diploids had pollen 66.5 to 85.5 microns in diameter.

Pollen from 28 of the 40 diploid plants was uniform in size and showed little or no abortion (Fig. 1 A) while 14 to 67 per cent of pollen from the other 12 plants was aborted. The tetraploids had from 67 to 78 per cent abortion (Fig. 1 C) while the triploids had 38 to 47 per cent aborted pollen (Fig. 1 E).

The chromosome number was determined for 28 seedlings from untreated seed of Spectrum Red ($N=5$). Among the 69 seedlings considered tetraploid, chromosome numbers ($N=10$) were determined for 22. Twenty of these and 20 diploid plants were propagated for further study. The three plants selected from the display bed were diploid ($N=5$). Treated tips which were rooted and later propagated by cuttings were tetraploid ($N=10$). Hybrids between tetraploids and diploids which will be discussed later were triploid with 15 chromosomes in the root tips.

Fertility studies. Diploid plants were highly self-fertile to fully controlled pollinations made in the greenhouse while all self- or close-pollinations on the tetraploids failed completely in seed production. This may be due to the high percentage of pollen abortion as noted above or to the failure of the pollen tubes to reach the ovary. Tetraploids crossed with diploids gave triploids, some of which had more viable pollen than the tetraploids but which also failed completely in the production of seed to controlled self-pollinations. All of the plants included in the present study were placed in the field in the summers of 1952-1955 during which time none of the tetraploids or triploids produced seed either to controlled- or open-pollinations. Tetraploids interplanted with diploids produced some viable seed from which triploid plants were obtained. Tetraploid cosmos have been reported to be fertile in the field while they were self-sterile when grown in the greenhouse (11). Since verbena polyploids remained sterile regardless of growing conditions thus far tested, viability of pollen and the rate of pollen-tube growth were investigated.

Pollen from most of the diploids when placed on an artificial medium and held in a moist chamber for 24 hours gave excellent germination (Fig. 1 B). A medium of 2 per cent agar and 20 per cent sucrose was found favorable for the germination of verbena pollen. Pollen from tetraploids was always scant. When placed on the same medium as used for diploid pollen, only an occasional tube was produced (Fig. 1 D). Pollen from triploids was more abundant. However, few tubes were seen on an artificial medium (Fig. 1 F). No attempt was made to find a more favorable

medium for the artificial germination of pollen from tetraploids and triploids.

After self-pollinations, the pollen tubes in the highly self-fertile plants of the diploid Spectrum Red began to grow soon after the pollen was placed on the stigma and after four hours had completed about one-sixth of the distance to the ovary (Fig. 2). The most rapid growth occurred from 8 to 12 hours after pollination, and many tubes were in the lower portion of the style near the ovary. One tube was found in a micropyle 14 hours

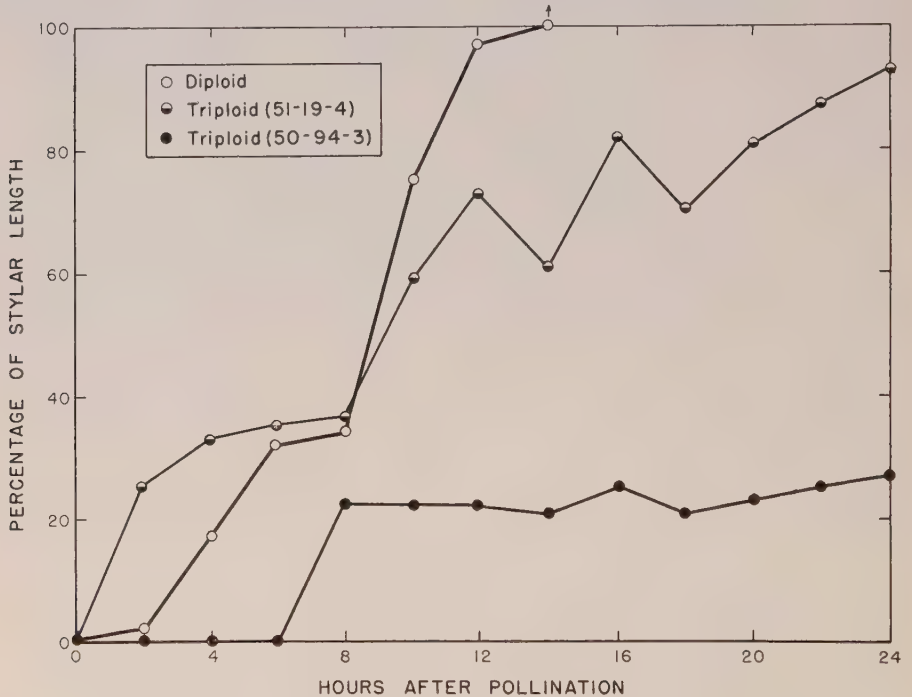


FIGURE 2. Curves for pollen-tube growth in three clones of verbena.

after pollination. After 16 hours more tubes had entered the ovary cavity and were seen in or near the micropyle of all four ovules. These tubes were straight and completed their growth through the style in a direct manner.

A similar study was made for two triploid clones which produced adequate pollen for such studies. These triploid clones have not set seed to fully controlled self-pollinations. In clone 50-94-3, produced by crossing the tetraploid of Spectrum Red with the original diploid type, germination was delayed for six hours. A few grains germinated and produced tubes which grew about one-fourth the distance to the ovary during the next two-hour interval (Fig. 2). After 48 hours when the pistil began to wilt,

the tubes had grown only 28 per cent of the distance required to reach the ovary. These short tubes assumed a devious course, had enlarged tips which burst, and exuded their contents within the style. The failure of this clone to set seed to self-pollination was due to the inability of pollen tubes to reach the ovary before the style withered. In the triploid clone 51-19-4 (purple tetraploid \times Spectrum Red diploid), pollen germinated soon after it was placed on the stigma and after two hours had grown one-fourth of the entire length of the style. The growth of pollen tubes in the various styles was somewhat erratic though a curve for the composite studies on growth shows a rather uniform increase in length of tubes from 2 to 24 hours after pollinations were made (Fig. 2), at which time a few tubes were seen near the base of the style but none were seen in the ovary cavity. Occasionally tubes did reach the ovary 30 and 48 hours after pollination. However, only one tube in one of the three pistils dissected for each of these two periods was observed in the ovary.

A study of the rate of growth of pollen tubes in the tetraploid was not feasible since pollen was scant due to much abortion. A few of the large grains filled with cytoplasm germinated on the stigma, but their course through the style was not determined.

General characteristics of polyploids. Polyploid verbenas exhibited the usual characteristics typical of induced polyploids. Increased vegetative vigor in leaves, flowers, and stems make polyploids very desirable plants for bedding or for use as cut flowers. Individual flowers of tetraploids were larger than those of diploids. The number of flowers per cluster was greater for the tetraploids (Fig. 3 B, E, I) than for the diploids (Fig. 3 A, D, G). Polyploids retained excellent vegetative growth and produced many flowers until killed by heavy frost.

Selections. Due to the increase in flower size, the larger number of flowers per cluster and excellent vegetative habit, five selections were made for commercial introduction. The following United States plant patents were issued December 21, 1954.

Tetra Red Verbena (plant patent 1,330) is characterized by the bright red flowers which according to Ridgway's Color Chart (12) are light carmine, merging into spectrum red toward the margin. According to Munsell's Color Standards (10), the flowers are R 3/12 (red hue, value 3, chroma 12) and merge to R 4/14 at the margin. The flower clusters are usually at least two and one-half inches in diameter and one and three-quarter inches deep. They are composed of 35 to 40 individual flowers which are about thirteen-sixteenths of an inch in diameter. The flower clusters are borne on stiff stems about one-eighth inch in diameter and usually at least 12 inches long. The leaves are thick, dark green in color, and are about one and one-fourth inches broad and about two inches long near the base while the leaves at the tips of the branches are smaller. It



FIGURE 3. Diploid and polyploid verbena: A, D, Diploid Spectrum Red; B, E, Tetraploid Spectrum Red; C, Triploid Purple; F, Triploid Rose Red; H, Tetraploid Rose; G, Diploid White; and I, Tetraploid White. A, B ($\times 0.75$); D, E, ($\times 0.70$); G, I ($\times 0.125$); and C, F, H ($\times 0.5$).

remains in good vegetative condition throughout the growing season and the leaves remain green and do not burn during the summer. The plant flowers well and has a profusion of bloom until killed by heavy frost. It has been propagated vegetatively without any difficulties.

Tetraploid Rose Verbena (plant patent 1,331) has exceptionally large flowers which are frequently over one inch in diameter. According to Ridgway's Color Chart, the color of the corolla is nearest Eugenia red with lighter areas toward the center of begonia rose (Fig. 3 H). In the young flowers the center of each flower is greenish-white. After Munsell the color is nearest Rp-R 5/10 (red purple red hue, 5 value, 10 chroma). There are generally 28 to 38 flowers in a single cluster which measures three to three and one-half inches in diameter. They are produced on stiff stems and are held about 13 inches above the ground. The leaves are large, thick, and dark green. They are as much as three and one-quarter inches long and two and one-quarter inches broad. A slight fragrance of the flowers may be detected.

Triplod Rose Red Verbena (plant patent 1,332). The bright rose-red color (Ridgway) of the flowers is distinct from other verbena flowers. The color is uniform throughout the corolla except for the young flowers which have a small white center which becomes less pronounced in older flowers (Fig. 3 F). According to Munsell's Book of Color, the color of the flowers is R 4/14 (red hue, 4 value, 14 chroma). Individual flowers are about one inch in diameter. An average of 30 flowers form clusters which measure two and one-half inches across and two inches in axial length. Flower clusters which are produced on stiff stems are usually 15 to 16 inches above the ground. Individual stems are from 16 to 23 inches long.

Triplod Purple Verbena (plant patent 1,333). This plant also has good vegetative vigor. Leaves are dark green and the lower ones are two and one-half X two and one-quarter inches. It produces large clusters of 40 to 50 flowers which are held about 12 inches above the ground. The individual flowers are about one inch in diameter and according to Ridgway's Color Chart are between prune purple and fluorite purple. The nearest color according to Munsell is P 3/8 (purple hue, 3 value, 8 chroma). The freshly opened flowers have a marked white eye which extends from the center of the corolla to the point of union of the petals which produces a star-like appearance (Fig. 3 C). The white area disappears in older flowers which are uniformly purple. The flowers have a noticeable but slight fragrance.

Triplod Magenta Verbena (plant patent 1,334) is characterized by its unique magenta flowers which are approximately three-fourths of an inch in diameter. The freshly opened flowers have white centers while the remainder of the corolla have colors according to Ridgway of near dahlia purple changing to Schoenfield's purple with age. According to Munsell's Color Standards the fresh flowers are RP 3/10 (red purple hue, value 3,

chroma 10) changing to RP 4/12 with age. The flowers have a strong fragrance. Flower clusters composed of 45 to 55 flowers are borne on stiff stems, many of which are 22 inches long, which hold the flower clusters erect, usually about 18 inches above the ground.

All five of these patented plants have been tested in field plantings as well as in home gardens. They have been of considerable horticultural interest and may serve to direct further attention to verbenas as popular garden plants.

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RESPIRATORY METABOLISM OF PREPUPAE AND PUPAE OF THE HOUSE FLY, *MUSCA DOMESTICA* L., AND OF THEIR HOMOGENATES¹

VAL F. COTTY

SUMMARY

Oxygen consumption of prepupae, pupae and their homogenates of the house fly, *Musca domestica* L., has been measured during metamorphosis. Oxygen consumption of intact insects plotted against time followed the U-shaped curve characteristic of other metamorphosing insects, whereas that of 10 per cent pupal homogenates did not. Endogenous oxygen consumption of homogenates was always less than that of intact pupae and was not increased by changes in tonicity or in pH of the medium at the levels studied. Sodium succinate, however, stimulated oxygen uptake in homogenates at the beginning and toward the end of metamorphosis and resulted in an oxygen uptake curve similar to that obtained with intact pupae. In the presence of succinate, cytochrome c caused further increases in oxygen consumption but alone it had no effect.

INTRODUCTION

The oxygen consumption of holometabolous insects follows a very definite pattern during metamorphosis. Krogh (13) first described a U-shaped respiratory curve as being characteristic of metamorphosis and many other investigators confirmed his observations (8, 9, 16, 22 and others). Wolsky (29) considered the changes in oxygen consumption to be related to the activity of the cytochrome system. This concept was supported by others who observed that the activity of cytochrome oxidase follows a U-shaped curve during metamorphosis (17, 22, 28). Agrell (1) attempted to correlate changes in respiration with alterations in various dehydrogenase systems. In a later paper (2), he stated that the ascending part of the U-shaped curve represents mainly an increasing activity of dehydrogenases in the thorax. He suggested that the descending arm of the curve is associated with the disintegration of the larval musculature and fat body, and the ascending arm with the development of imaginal musculature. Levenbook (15) demonstrated that the easily hydrolyzable phosphorus also follows a curve approximately U-shaped during the pupal stage, while inorganic phosphorus shows the opposite trend, being highest about half-way through the stage. He suggested that oxidative phosphorylation may be inhibited during the early part of metamorphosis.

In the present study tissue homogenates were used to measure the apparent levels of succinoxidase activity at various times during metamor-

¹ Accepted by the Faculty of the Graduate School of Arts and Science of New York University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The author wishes to express his deep appreciation for the interest, assistance and helpful criticism of Dr. Daniel Ludwig, under whose direction the work was conducted.

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phosis of house fly pupae in order to determine whether or not the variations in this activity can be correlated with variations in oxygen consumption of intact pupae.

MATERIALS AND METHODS

House flies (*Musca domestica* L.) of two different laboratory strains were used for the study. One was obtained from Dr. D. Ludwig of Fordham University and the other from Dr. A. Hartzell of the Boyce Thompson Institute for Plant Research, Inc. The adults were kept at room temperature (approximately 25° C.) and fed milk diluted with an equal volume of water. Eggs were deposited upon paper wicks placed in the milk. They were removed daily and 200 to 300 placed on the larval medium in a quart container. Two media were used. One was an agar medium containing 15 g. wheat germ, 15 g. powdered milk, 3 g. agar agar, 1 g. Brewer's yeast, 2.7 ml. 10 per cent Moldex in 95 per cent ethanol and 100 ml. H₂O. This medium was prepared as much as a week in advance and was autoclaved for 10 to 15 minutes at 15 lbs. pressure. The second medium consisted of rodent food pellets (manufactured by Rockland Farms, Inc.) fortified with wheat germ (1 part to 8 parts of medium) and soaked in tap water. This latter medium was very simple to prepare and gave excellent results. About 250 ml. of media were placed in each flask. The larvae and pupae were incubated at 25° C. When the larvae started to leave the food, they were removed from the container and placed in finger bowls filled with small pieces of paper toweling. As the larvae shortened and became immobile, they were removed from the dishes and were either used immediately or placed in beakers lined with paper toweling to absorb the moisture. The prepupae with white puparia were designated "o" age. Prepupae employed in respiration measurements were used within 30 minutes of puparium formation. There is considerable variation in the time of emergence of adult flies from the puparia. Hence material taken on the last day of the pupal stage is very variable. To eliminate the effect of this variability on the respiratory readings pupae were used up to four days after puparium formation.

All respiratory studies were made with Warburg constant volume respirometers. Oxygen consumption and carbon dioxide production of intact pupae and prepupae were measured by a modification of the method of Dickens and Simer as given by Levenbook (14). Four weighed insects were placed in the dry center well of each Warburg flask. The main compartment contained 2 ml. of 0.2 *M* potassium permanganate in 0.002 *M* H₂SO₄ and the side arm contained 0.2 ml. of 30 per cent sodium iodide acidified to 0.002 *M* H₂SO₄ immediately before use. The flasks were placed in the water bath at 30° C. and were shaken at 120 times per minute. After a 10-minute equilibration period the manometers were closed and readings were made every 15 minutes for 90 minutes. At 90 minutes the

contents of the side arm were tipped into the main compartment and 5 minutes later the final reading was made. Oxygen consumption and respiratory quotients were calculated according to Levenbook (14).

Oxygen consumption of homogenates was determined according to the following procedure. From 20 to over 100 pupae were placed in a small beaker and a few ml. of water were added with gentle stirring. One-half minute later approximately 4 volumes of 95 per cent ethanol were added to remove surface bacteria which were found to affect oxygen consumption. After stirring gently for two minutes the alcohol was poured off and the pupae were quickly but gently dried by blotting on paper toweling. The washed insects were permitted to dry thoroughly and were then weighed on an analytical balance and placed in a Ten Broek soft-tissue grinder. The desired amount of suspending medium to give a 10 per cent (w/v) homogenate was added and the insects were thoroughly homogenized by hand. The tissue grinder was kept cold by immersion in an ice bath and the homogenate was left in the ice bath until it was pipetted into the manometer flasks. In experiments on the effects of osmotic pressure on oxygen consumption, homogenates were suspended in solutions containing 1.4 per cent NaCl in 0.02 *M* phosphate buffer, 1 Molal sucrose in 0.02 *M* phosphate buffer, or in 0.02 *M* phosphate buffer alone. In each case the pH was adjusted to 7.0. In other experiments 10 per cent homogenates were prepared in 0.02 *M* phosphate at pH 7.0, or when other solutions were to be added, homogenate and buffer were prepared in higher concentrations so that the final concentrations were 10 per cent and 0.02 *M* respectively. The homogenate, with or without additions, was placed in the main compartment of the Warburg flasks. The center well contained 0.2 ml. of 10 per cent KOH and a pleated piece of filter paper to increase the surface of the KOH solution. The main compartment usually contained 1 ml. of homogenate except in the case of dilute homogenates when 3 ml. were used. Both 7 and 15 ml. flasks were used.

Sodium succinate, "crystal pure" grade, was obtained from Fisher Scientific Company and cytochrome *c* from Sigma Chemical Company or from Nutritional Biochemicals Corporation. All other chemicals were C. P. grade or purer.

RESULTS

During the period covered in this study, the oxygen consumption of intact insects followed the typical U-shaped curve as shown in Figure 1. In this figure is also plotted the endogenous oxygen consumption of 10 per cent homogenates suspended in 0.02 *M* phosphate buffer at pH 7.0. The homogenate curve was lower and flatter than that for the intact pupae and, in addition, was without the typical U-shaped profile. Figure 2 shows the endogenous respiration of the homogenates in terms of per cent respiration of intact insects. The respiration at the low part of the U-shaped curve was

much less susceptible to disruption by homogenization. The respiration of prepupae was reduced more than 80 per cent by homogenization, while that of one-day pupae was reduced only 40 per cent. Respiratory quotients are also plotted in Figure 2. They vary between 0.7 and 0.75 throughout the pupal stage.

Figure 3 shows a comparison of the endogenous respiration of 10 per cent homogenates in 0.02 *M* phosphate buffer at pH 7.0 and that of 10 per cent homogenate in Belar's solution, without calcium and glucose, but buffered with 0.02 *M* phosphate at a pH of 7.0. Differences are small ex-

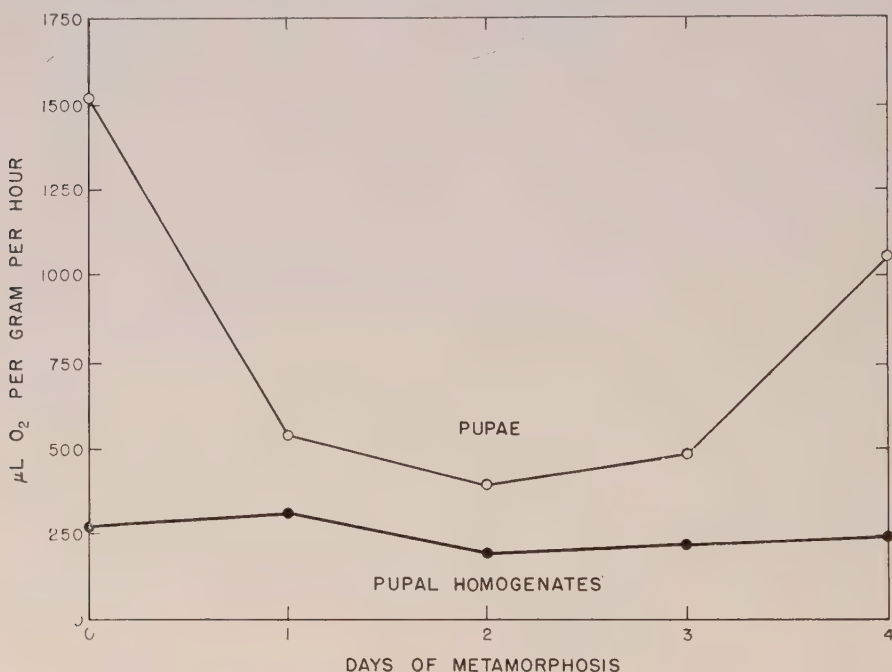


FIGURE 1. Oxygen consumption of intact pupae and of pupal homogenates during metamorphosis.

cept for one-day homogenates which show a considerably higher respiration in dilute buffer.

There were no differences in the oxygen uptake of four-day pupal homogenates suspended in 1.4 per cent NaCl in 0.02 *M* phosphate buffer, 1 Molal sucrose in 0.02 *M* phosphate buffer, or in 0.02 *M* phosphate buffer alone. Neither were there any differences when the homogenates were suspended in 0.02 *M* phosphate buffer at pH 6.5, 7.0 or 7.5. Ten per cent homogenates prepared from pupae immersed in water for prolonged periods of time previous to homogenization had an increased endogenous oxygen consumption. This increase is probably due to an accumulation of substrates during immersion. Such a substrate accumulation might occur

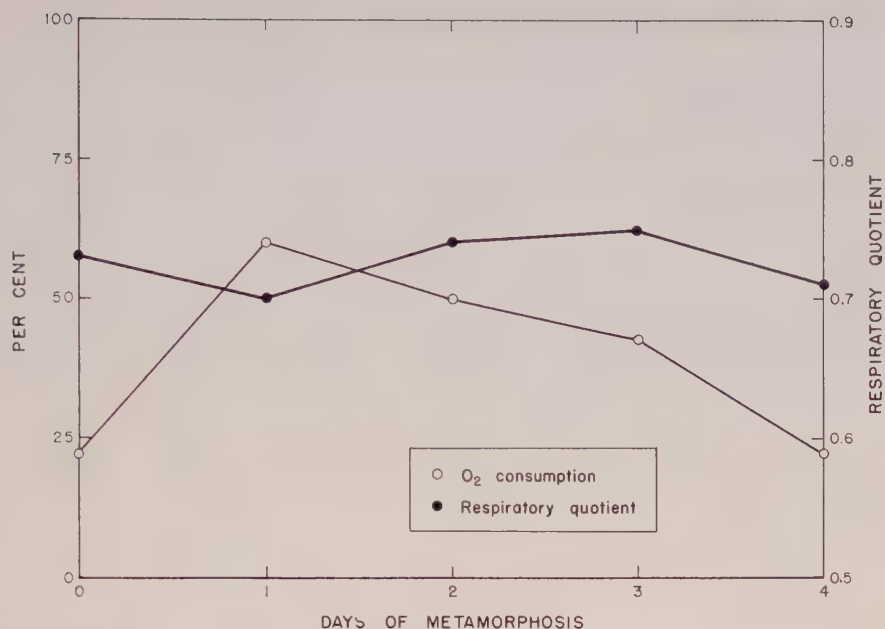


FIGURE 2. The effect of homogenization and variations in the respiratory quotient during metamorphosis (oxygen consumption of homogenates expressed as per cent of the value obtained for intact insects).

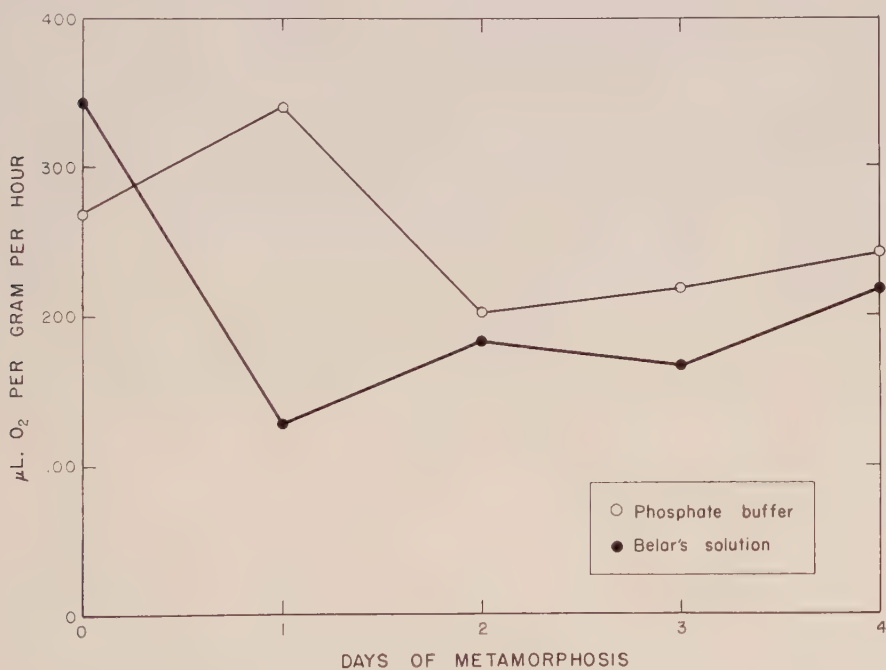


FIGURE 3. Oxygen consumption of homogenates suspended in Belar's solution or in 0.02 M phosphate buffer.

when respiratory exchange is blocked. The effect of $0.01\text{ }M$ disodium malonate was tested on 10 per cent pupal homogenates at several stages of metamorphosis. No inhibition was indicated for the stages studied.

The effects of $10^{-4}\text{ }M$ cytochrome *c* and $0.1\text{ }M$ sodium succinate on the oxygen consumption of 10 per cent homogenates are shown in Figure 4. Cytochrome *c* alone did not increase respiration in any case. Succinate inhibited respiration of homogenates from one-day pupae but stimulated respiration of 10 per cent homogenates of pupae at 0, 3 and 4 days after puparium formation. Hence, the respiratory curve of homogenates in the

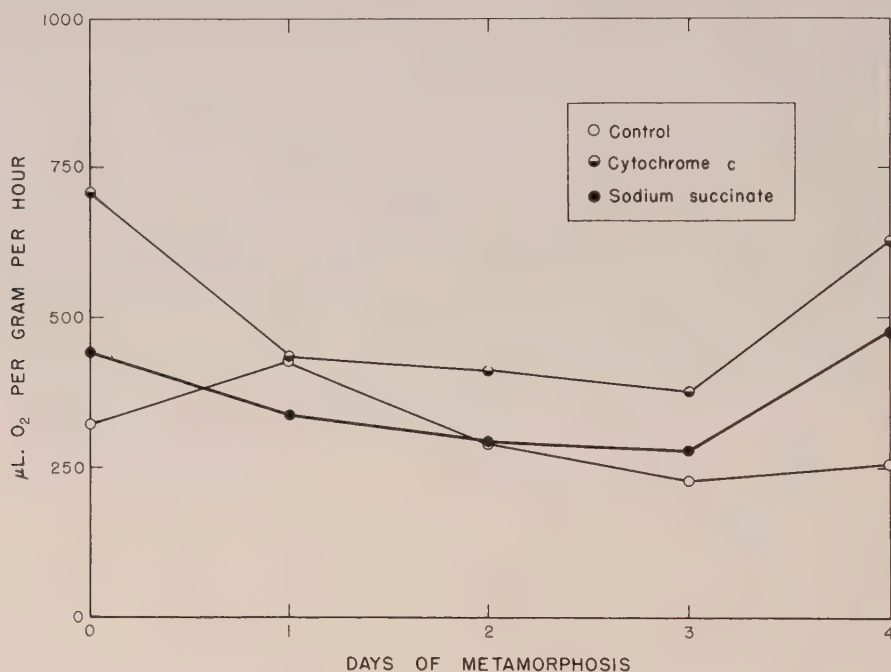


FIGURE 4. The effects of added cytochrome *c* ($10^{-4}\text{ }M$) and sodium succinate ($0.1\text{ }M$) on the oxygen consumption of homogenates during metamorphosis.

presence of succinate had the U-shaped profile typical of the respiration of the intact insects. Respiration was further increased when cytochrome *c* was present in addition to succinate.

One per cent homogenates in $0.02\text{ }M$ phosphate buffer had a lower endogenous oxygen consumption than that of 10 per cent homogenates but a much higher oxygen consumption in the presence of $10^{-4}\text{ }M$ cytochrome *c* and $0.1\text{ }M$ sodium succinate. Oxygen consumption of 1 per cent homogenates in the presence of cytochrome *c* and succinate was not significantly different at pH 7.4 from that at pH 7.0. The readings obtained on the effects of $0.25\text{ }M$ sucrose on pupal respiration were not consistent. Respira-

tion of 1 per cent homogenates was higher in the presence of succinate when the suspending medium was a dilute buffer than when the medium was 0.25 *M* sucrose in buffer. The situation was reversed when 3.3 per cent homogenates were employed.

DISCUSSION

The results obtained with pupal homogenates of the house fly agree with those of Bodine (3) in that the respiration of the intact animal is always greater than the endogenous respiration of homogenates. However, Bodine obtained a decrease of 65 per cent in respiration for both active and blocked (or diapause) *Melanoplus differentialis* embryos; while in the present work, the decrease resulting from homogenization varied throughout metamorphosis. Bodine and Lu (4) stated that this loss was due to "structure." They did not take into account dilution by the suspending medium nor the possible disappearance of essential substances in the homogenates. They reported a 100 per cent increase in O_2 uptake upon addition of 10^{-5} *M* dinitrophenol (DNP) to intact embryos. This increase in O_2 uptake was presumably due to uncoupling of phosphorylation from oxidation (10). Since DNP did not affect O_2 uptake in homogenates it seems likely that homogenization resulted in inactivation of that part of the oxidation mechanism concerned with oxidative phosphorylation.

Sacktor² suggested that perhaps homogenization could cause several different factors, including cytochrome c and available substrate to limit oxygen consumption. In the present tests cytochrome c produced no stimulation but added succinate increased oxygen consumption considerably at the beginning and at the end of metamorphosis. This observation suggests that substrate availability might be a factor involved in the reduction of the U-shaped oxygen consumption curve of intact pupae to the much lower flat curve of pupal homogenates. However, only succinate was tested as a substrate. Since diphosphopyridine nucleotides are rapidly broken down by tissue homogenates (11), it is possible that inactivation of enzymes requiring these coenzymes also might be a factor in the loss of oxidative activity. The fact that cytochrome c increased oxidative activity in the presence of succinate suggests that some cytochrome c was lost. Loss of cytochromes during homogenization frequently occurs (24) and undoubtedly contributes to loss of oxidative activity. Thus the flat respiratory curve obtained with pupal homogenates occurs as a result of many factors.

The fact that isotonic saline (1.4%) and hypertonic sucrose produced no effect on the endogenous respiration of homogenates suggests a difference from the condition existing in *Melanoplus differentialis* embryos. In homogenates of these grasshopper embryos suspended in 0.25 *M* sucrose, there was a lower endogenous respiration (5) than that of homogenates

² Personal communication.

suspended in Ringer's solution. Homogenates in 0.25 *M* sucrose containing succinate showed a very much greater oxygen consumption than that of homogenates in Ringer's solution with succinate. Here again there is no agreement with results obtained on the house fly. Schneider, Claude and Hogeboom (25) showed that a sucrose suspending medium, both isotonic and hypertonic, preserved the structure and function of mitochondria. This observation may not apply to the house fly, since Sacktor (23) showed that solutions isotonic for house fly sarcosomes must contain, in addition to sucrose, an inactive protein to prevent degenerative changes. This observation corroborates the work of Watanabe and Williams (27) on sarcosomes of other Diptera. The absence of any inhibition of endogenous respiration with 0.01 *M* malonate is in agreement with the results of Bodine, Lu and West (6).

Needham (20, p. 473), referring to Wolsky's work (29), stated it might be expected, on the basis of an analogy with embryonic diapause, that respiration during the low part of the U-shaped curve would be insensitive to carbon monoxide. However, while succinoxidase activity appears to pass through a very low minimum during metamorphosis, its greatest activity is present during embryonic diapause (7). Thus there is a great difference between the two physiological states, diapause and metamorphosis. When diapause occurs during the pupal stage there may result a rather complicated condition which may not be the same as metamorphosis without a diapause.

The relatively little effect of homogenization, the lack of stimulation by succinate and the insensitivity to cyanide (22), which characterize respiration at the lower portion of the U-shaped curve, argue strongly for changes in the kinds of terminal oxidases responsible for O₂ uptake. Since the activity of the cytochrome oxidase is at a low level in one- and two-day pupae, enzymes similar to xanthine oxidase (19) with the ability to catalyze the oxidation of a large number of different substrates produced by extensive lysis of larval tissues might be present. The large cyanide insensitive fraction of respiration during metamorphosis may be due to a relative increase in the activity of aerobic dehydrogenases, especially those concerned with nitrogen metabolism.

Shappirio and Williams (26) showed that cytochrome *e* is probably the terminal oxidase of the cyanide- and CO-insensitive tissues of the diapausing *Cecropia* pupa. Pappenheimer and Williams (21) stated that in the midgut a considerable portion of the total hydrogen transport passes from DPNH to cytochrome *e*. They suggested that though cytochrome *e* undergoes auto-oxidation more slowly than cytochrome oxidase it can act as a terminal oxidase. They reported that this cytochrome is unaffected by cyanide, carbon monoxide or antimycin A. However, the oxidation of succinate may not involve cytochrome *e*. The possible significance of this system in nondiapause pupae has not been determined.

It was found that the endogenous oxygen consumption of 1 per cent homogenates is lower than that of 10 per cent homogenates while the oxygen consumption in the presence of succinate and cytochrome c is much higher. A possible explanation may be that not only are enzymes and substrates diluted, but the concentration of possible inhibitors (such as oxalacetate which is a strong inhibitor of succinoxidase) is also reduced.

The inhibition of endogenous respiration in 10 per cent homogenates of one-day pupae by 0.1 *M* sodium succinate is more difficult to explain. At this stage there is very little cytochrome oxidase present (22). It is likely that some aerobic dehydrogenase, which is relatively important at this time, is inhibited. An example of an enzyme with similar behavior is D-amino acid dehydrogenase which is inhibited by sodium chloride. It is interesting to note that homogenates of one-day pupae have a much lower oxygen consumption when suspended in Belar's solution than in dilute buffer. The chief salt present in Belar's solution is sodium chloride.

The results reported in the present work are in general agreement with those of Ludwig and Barsa³ on *Tenebrio* pupae, differences in most cases being quantitative. They also found that homogenization resulted in a disappearance of the U-shaped curve and that the addition of cytochrome c and sodium succinate stimulated respiration of homogenates. However, one departure from the results obtained with house fly pupae was the marked stimulation of endogenous respiration when cytochrome c was added to homogenates of the mealworm. They (18) found that succinoxidase activity was low during metamorphosis, rising sharply at the end. This finding is in agreement with those Ito (12) obtained with the silkworm. In the present study, the U-shaped respiratory curve of homogenates in the presence of succinate indicated that succinoxidase activity dropped from an initial high level to a minimum and then rose again toward the end of the metamorphic period.

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SUSCEPTIBILITY OF PLANTS TO HYDROFLUORIC ACID AND SULFUR DIOXIDE GASES

P. W. ZIMMERMAN AND A. E. HITCHCOCK

SUMMARY

The effects of hydrofluoric acid (HF) and sulfur dioxide (SO₂) gases were determined and compared on 49 species of plants. At given concentrations and periods of exposure some species of plants were susceptible to HF but resistant to SO₂, and some species susceptible to SO₂ were resistant to HF. A few species were equally susceptible to both gases, and some were equally resistant. In tests with HF alone, concentrations of the gas ranged from less than 1 to 100 parts per billion (10⁹), by volume, and periods of exposure were usually six to eight days. The concentration for SO₂ gas ranged from 0.2 to 1.1 part per million (10⁶), and the time periods for exposure to the gas were two to eight hours. Special equipment and methods were developed for fumigating plants in the field and in greenhouses.

Stomatal counts for upper and lower epidermis of leaves showed no relation between the number of stomata per unit leaf area and relative susceptibility to either gas.

All species of plants grown in pots or under field conditions stored fluorine. A few like dogwood (*Cornus florida* L.), *Deutzia* sp., gifblaar (*Dichapetalum cymosum* Hook.), and *Camellia* sp. accumulated abnormally large amounts of fluorine in the leaves. There was considerable difference in rate of absorption by different species when the plants were fumigated, and different parts of the plant absorbed different amounts of fluoride.

These results should serve as an aid in diagnosing symptoms on vegetation around industrial areas.

INTRODUCTION

Plant species vary in their expression of symptoms when exposed to different gases in the air. In some cases the symptoms are unique enough so that the effective agent can be identified (11). The variation in susceptibility, together with the symptoms involved, aids in a study of air pollution. For example, corn becomes mottled when exposed to HF but not SO₂, while Spanish needles is resistant to HF but susceptible to SO₂. In order to induce symptoms associated with well-known air pollutants and mixtures of these, special equipment was built for dispensing the chemicals and fumigating plants with known concentrations of the various gases. In his review of gas damage to plants Thomas (10) described the effects of several gases.

This report concerns the effects of fluorides and sulfur dioxide when applied to a large number of species of plants. Methods and equipment used in the experiments are also described. A study was made of the number of stomata in leaves of many species of plants.

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MATERIALS AND METHODS

Both portable greenhouses and stationary cabinets were used as fumigation chambers for experimental plants. The portable houses were especially useful for treating plants which were growing in the field. They are illustrated in Figure 1. Two stationary cabinets are located within a heated greenhouse for year-round work. Temperature in the stationary cabinets was controlled during the winter by means of electrically-regulated steam space heaters located in the air ducts leading to the cabinets. Provision was made to supply the cabinets with air drawn from outside or inside the greenhouse. The equipment for dispensing the fluorides in the stationary cabinets is essentially the same as that described for the portable houses. However, certain mechanical changes were required to adapt the fluoride dispensing equipment for use with portable houses.

Portable greenhouses. Portable greenhouses were constructed of a cypress frame covered with Vinylite having a thickness of 0.004 inch. The Vinylite was stretched taut and tacked to the inside of the walls and to the outer top side of the roof, being further held in place by narrow strips of wood. A metal stack 15 inches in diameter was located in the center of the roof to carry off the effluent gases.

The base of the walls rested on and was bolted to a rectangular wooden duct 10 ft. square which constituted the base of the house. The inside cross-sectional dimension of the wooden duct was 9.25×11.0 inches (0.706 sq. ft.). A short metal duct was used to conduct air from the blower to the rectangular opening located in the center of one side of the wooden duct. Regularly spaced openings on the inside walls of the wooden duct permitted distribution of the incoming air with sufficient turbulence to insure mixing before being blown out of the exit stack in the roof.

All surfaces of the wood were painted with two coats of white paint and then with a coat of paraffin. Metal surfaces were painted with red lead, aluminum paint, and, finally, the inside of the ducts was coated with paraffin.

Inside dimensions of the greenhouse were as follows: wall height 7 ft. from ground level, width of each wall 8 ft., and peak of the gable 1.5 ft. The total volume was approximately 500 cu. ft. A hinged door 6×2.5 ft. was placed on the side opposite to that used for the air intake.

Air blower. A slip fan type of blower with a capacity to deliver up to 2000 cu. ft. per minute was used to propel air into the greenhouse (Fig. 1 A). The rate of air flow could be regulated by a blast gate located on the delivery side of the fan so as to deliver 1 to 5 air exchanges per minute. The rate of air flow used in most tests was 1.5 air exchanges per minute. The blower and other motors were provided with weatherproof equipment.

Air scrubber and humidifier. Air was drawn through a scrubber (Fig. 1 A) before being blown into the greenhouse. The scrubber consisted of a



FIGURE 1. Portable greenhouses covered with Vinylite used for fumigating field-grown and potted plants. A. Close-up view of one house showing in foreground, right to left, scrubber unit, air blower, and weatherproof box containing gas injection equipment and automatic air sampling unit. B. View of four houses used for fumigating plants with HF gas injected into scrubbed air and two control houses used for plants receiving scrubbed air only.

two-piece metal box inside of which were eight nozzles that delivered an atomized water spray directed toward the incoming air. The nozzles were mounted in two vertical pipes which were supplied with water under pressure by means of a recirculating pump. The nozzle equipment, recirculating pump, and a strainer on the inlet water pipe were attached to the lower part of the box which consisted of a tray holding 10 inches of water. The upper part of the box consisted of a removable cover provided at each end with slanted baffles. A hose bib located at the base of the scrubber unit was used for draining the tray. The scrubber unit was coupled to the blower by means of a removable metal reducing sleeve as shown in Figure 1 A.

Gas injection equipment. Several methods have been used at Boyce Thompson Institute for injecting fluoride gases into fumigation chambers (3). The method finally adopted in 1954 makes use of atomization and thermal action. A recent improvement (1955) involves the use of platinum-tipped nozzles instead of the ones furnished with No. 15 DeVilbiss atomizers (3).

The concentration of fluoride gas measured in the stationary cabinets and portable greenhouses was proportional in the range 0.6 to 100 parts per billion (p.p.b.) to the percentage concentration of fluosilicic acid used (0.01 to 6.0 per cent). The fluosilicic acid was prepared from a commercial 30 per cent reagent and the HF acid from a 48 per cent reagent. However, since the opening in different platinum nozzle tips was not the same, each nozzle had to be calibrated for use with a given fumigation chamber. When it was desirable to eliminate silicon tetrafluoride, polyethylene dispensing equipment with HF solutions were used instead of glass equipment and fluosilicic acid solutions. The injection equipment (3) and automatic sampling unit (4) were placed in a weatherproof box (Fig. 1) when used in the field. Metered samples of air containing volatile fluorides were collected and analyzed as described previously (4).

SO₂ was supplied as a mixture of 10 per cent SO₂ and 90 per cent dry nitrogen contained under pressure in a steel storage cylinder. The SO₂ was injected through Tygon tubing into the air ducts leading to the fumigation cabinets. Pressure was controlled by an SO₂-resistant pressure regulator attached to the outlet of the storage cylinder. The rate of flow was controlled by a glass flow meter provided with interchangeable capillary glass tubes of different length and different bore diameter. Ground glass joints at one end of the capillary tubes insured a tight connection and facilitated the exchange of capillary tubes.

The concentration of SO₂ in fumigation chambers was determined by the peroxide-alkali titration method (2, p. 41) except that a borax solution was used instead of sodium hydroxide or sodium carbonate (8, p. 541). This method was compared periodically with the iodine titration method (12), and in all instances the results showed close agreement. Metered

samples of the air-SO₂ mixtures in the fumigation cabinets were collected in closed type, conical-shaped absorbers as described for the collection of samples of air containing volatile fluorides (4).

Mixing of injected HF with air in greenhouse. The uniformity of HF-marked leaves on corn and gladiolus plants occupying different positions in the treated plot indicated that the injected HF gas was thoroughly mixed with the air forced through the greenhouse. These results show that the method of delivering the incoming HF-air mixture at the base of the greenhouse, and providing for the escape of effluent gases through a centrally located stack in the roof, was satisfactory for fumigating the kinds of plants used.

Some of the species and varieties of plants used are listed in Tables I, II, and III. With relatively low concentrations of HF gas (less than 1 to 5 p.p.b.) the exposure usually lasted seven days or more. With a concentration of approximately 100 p.p.b. of the gas, a period of 24 to 48 hrs. was sufficient to bring out the symptoms. When SO₂ was involved, the exposures usually lasted 4 to 24 hrs. at 500 p.p.b. Special experiments were conducted for 4 to 8 hrs. with SO₂ at 500 p.p.b. [0.5 parts per million (p.p.m.)] and HF at 50 p.p.b.

The fluorine content of plant tissue was determined by methods previously reported (5, 6).

EXPERIMENTAL RESULTS AND DISCUSSION

Approximately 40 species of plants were exposed under similar conditions to SO₂ and HF gases. The purpose of this experiment was to determine the comparative susceptibility of species to the two gases (Table I). It was desired to bring out characteristic markings within four- to eight-hour periods. Since the gases were not equally toxic, different concentrations of the two gases had to be used. After making a number of preliminary tests at various concentrations of the gases, 50 p.p.b. was selected for HF and 500 p.p.b. for SO₂ gas. With these concentrations it was possible to compare species which were highly susceptible or resistant and those which were of medium sensitivity. The results are tabulated in Table I. The characteristic symptoms induced by SO₂ and HF gases on several species of plants are illustrated in Figure 2. Variation in comparative susceptibility occurred with different length of the periods of exposure, and age or condition of the plant.

For some unknown reason the species of plants most susceptible to HF, such as Jerusalem cherry, gladiolus, tulip, maize, ixora, and corn, were resistant to SO₂ (Tables I and II). Several species most susceptible to SO₂, chicory, eggplant, geranium, pigweed, tobacco, dandelion, and celery, were resistant to HF. Cotton and stevia were comparatively resistant to both gases. Italian prune, buckwheat, smartweed, wild and cultivated varieties

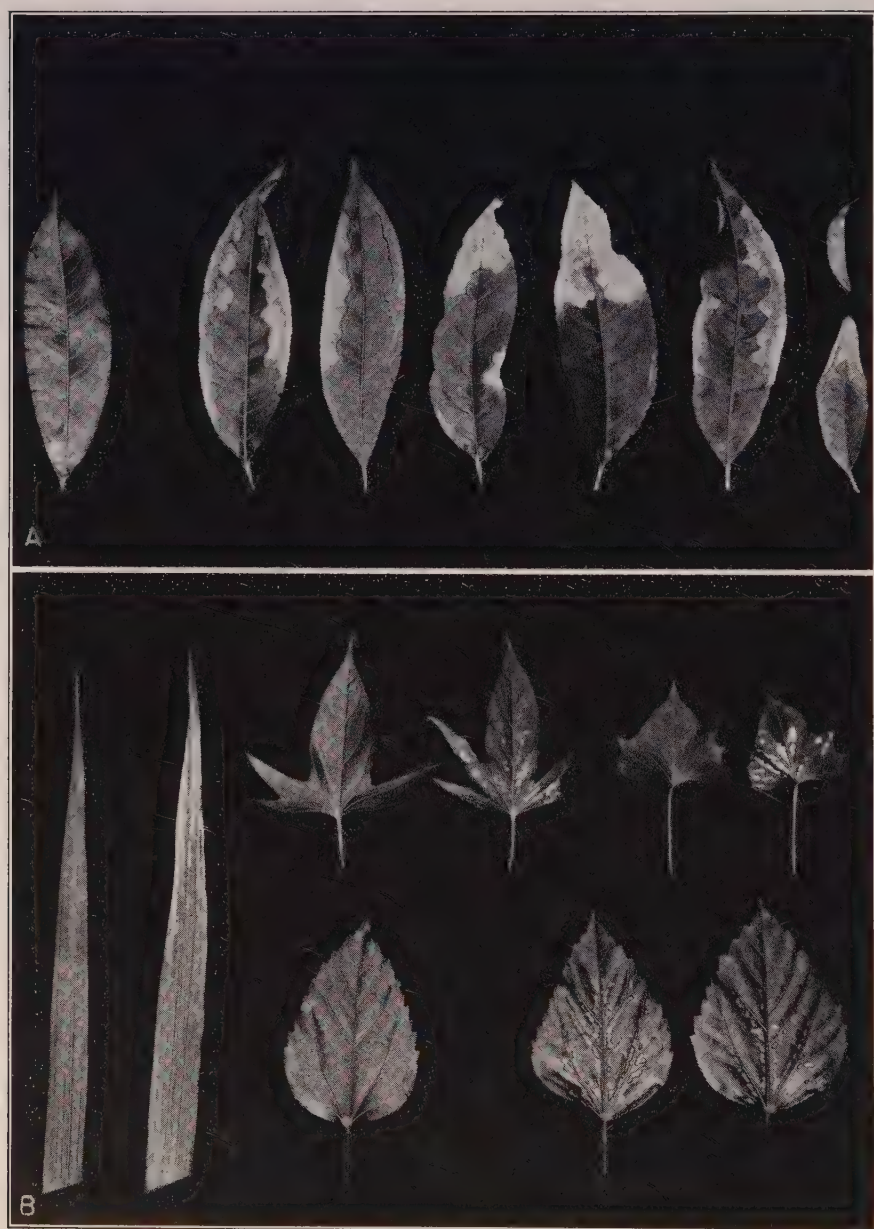


FIGURE 2. Symptoms induced by HF and SO_2 gases. Control leaf of each variety is at left in each group. A. Peach leaves showing variation from exposure to 10 to 14 p.p.b. HF gas for seven days. B. Leaves of plants exposed nine days to 900 p.p.b. SO_2 gas. Left, gladiolus; upper row, two varieties of sweet potato; lower row, tropical *Hibiscus*.

TABLE I

COMPARATIVE SUSCEPTIBILITY OF PLANT SPECIES EXPOSED FOUR TO EIGHT HOURS TO APPROXIMATELY 500 P.P.B. SO₂ AND 50 P.P.B. OF HF GASES AND THE STOMATAL COUNTS ON THE UPPER AND LOWER SURFACES OF LEAVES. THE SPECIES ARE ARRANGED ACCORDING TO DECREASING SENSITIVITY TO HF GAS

| Species | Susceptibility to gases* | | No. of stomata per sq. mm. in epidermis | |
|---|--------------------------|-----------------|---|-------|
| | HF | SO ₂ | Upper | Lower |
| Jerusalem cherry, <i>Solanum Pseudo-Capsicum</i> L. | XXXX | o | 73 | 374 |
| Gladiolus vars., <i>Gladiolus</i> sp. | XXXX | X | 187 | 189 |
| Tulip, <i>Tulipa</i> sp. | XXXX | o | 58 | 36 |
| Maize, milo, <i>Sorghum</i> sp. | XXX | o | 66 | 187 |
| Ixora, <i>Ixora</i> sp. | XXX | o | o | 326 |
| Corn vars., field, <i>Zea mays</i> L. | XXX | o | 55 | 70 |
| Apricot, Moorpark, <i>Prunus</i> sp. | XXX | X | o | 235 |
| Prune, Italian, <i>Prunus</i> sp. | XXX | XXX | o | 240 |
| Buckwheat, <i>Fagopyrum esculentum</i> Moench. | XX | XXX | 45 | 145 |
| Smartweed, perennial, <i>Polygonum</i> sp. | XX | XX | 23 | 99 |
| Grape, wild, <i>Vitis labrusca</i> L. | XX | XX | — | — |
| Corn vars., sweet, <i>Zea mays</i> L. | XX | o | 46 | 94 |
| Sweet potato, <i>Ipomoea batatas</i> Lam. | XX | XXX | 26 | 26 |
| Iris, <i>Iris</i> sp. var. Great Lakes | XX | X | 43 | 45 |
| Apple, <i>Malus</i> sp. | XX | X | o | 219 |
| Rose, <i>Rosa</i> sp. | X | XX | o | 96 |
| Lamb's-quarters, <i>Chenopodium album</i> L. | X | XX | 115 | 189 |
| Dock, curly, <i>Rumex</i> sp. | X | XXX | 23 | 104 |
| Stevia, <i>Piqueria trinervia</i> Cav. | o | o | 10 | 90 |
| Cotton, <i>Gossypium hirsutum</i> L. | o | X | 53 | 134 |
| Tobacco, Turkish, <i>Nicotiana tabacum</i> L. | o | XXX | 83 | 137 |
| Taxus, <i>Taxus</i> sp. | o | XX | o | 106 |
| Bean, <i>Vicia</i> sp. | o | XX | 47 | 178 |
| Chicory, escarole, <i>Cichorium</i> sp. | o | XXXX | 42 | 81 |
| Dandelion, <i>Taraxacum officinale</i> Weber | o | XXX | 121 | 165 |
| Spanish needles, <i>Bidens</i> sp. | o | XXX | 79 | 360 |
| Nightshade, <i>Solanum nigrum</i> L. | o | XXX | — | — |
| Celery, <i>Apium graveolens</i> L. | o | XXX | 101 | 158 |
| Tomato, <i>Lycopersicon esculentum</i> Mill. | o | XXX | 32 | 112 |
| Pumpkin, <i>Cucurbita pepo</i> L. | o | XXX | — | — |
| Cucumber, <i>Cucurbita sativus</i> L. | o | XXX | — | — |
| Pigweed, <i>Amaranthus retroflexus</i> L. | o | XXX | — | — |
| Alfalfa, <i>Medicago sativa</i> L. | o | XXXX | 113 | 124 |
| Clover, sweet, <i>Melilotus</i> sp. | o | XXX | 420 | 208 |
| Coleus, <i>Coleus blumei</i> Benth. | o | XXX | 64 | 188 |
| Geranium, <i>Pelargonium</i> sp. | o | XXXX | 33 | 175 |
| Buttonbush, <i>Cephalanthus</i> sp. | o | XXXX | o | 242 |
| Eggplant, <i>Solanum melongena</i> L. | o | XXXX | — | — |
| Galinsoga, <i>Galinsoga parviflora</i> Cav. | o | XX | 1 | 55 |

* o=no markings; X=slight markings; XX to XXXX=moderate to pronounced markings.

of grape, and sweet potato, were approximately equally susceptible to both gases.

The symptoms associated with exposure to SO₂ and HF are different enough so that when several of the same species exposed to the gases were examined and compared, the causal agent could be identified. Corn, for

TABLE II

PLANT SPECIES NOT LISTED IN TABLE I SHOWING STOMATAL COUNTS AND RELATIVE SUSCEPTIBILITY TO HF AND SO₂ GASES. THE CONCENTRATION OF THE GASES IN DIFFERENT TESTS RANGED FROM LESS THAN 1 TO 100 P.P.B. HF AND 0.1 TO 1 P.P.M. SO₂. PERIODS OF EXPOSURE TO THE GAS WERE ONE TO EIGHT DAYS FOR HF AND TWO TO EIGHT HOURS FOR SO₂

| Species | Relative susceptibility to gases* | | No. of stomata per sq. mm. in epidermis | |
|--|-----------------------------------|-----------------|---|-------|
| | HF | SO ₂ | Upper | Lower |
| Azalea, <i>Rhododendron obtusum</i> Planch. var. <i>Hinodegiri</i> | I | I | 0 | 201 |
| Begonia, <i>Begonia</i> sp. | I | — | 0 | 80 |
| Blueberry, <i>Vaccinium corymbosum</i> L. | S-I | S | 0 | 302 |
| Chicory, endive, <i>Cichorium</i> sp. | R | S | — | — |
| Chicory, San Pasquale dandelion, <i>Cichorium</i> sp. | R | S | — | — |
| Cinquefoil, <i>Potentilla</i> sp. | R | — | 40 | 214 |
| Deutzia, <i>Deutzia</i> sp. | I | R | 0 | 94 |
| Grape, <i>Vitis</i> sp. var. <i>Carignane</i> | S | — | 0 | 182 |
| Grape, <i>Vitis</i> sp. var. <i>Muscat</i> of Alexandria | S | — | 0 | 116 |
| Grape, <i>Vitis</i> sp. var. <i>Pedro Ximenes</i> | S | — | 0 | 199 |
| Grape, <i>Vitis</i> sp. var. <i>Salvador</i> | S | — | 0 | 268 |
| Hibiscus, <i>Hibiscus rosa-sinensis</i> L. | R | S | 8 | 242 |
| Honeysuckle, bush, <i>Lonicera</i> sp. | I | I | 0 | 324 |
| Hypericum, <i>Hypericum</i> sp. | S | — | 26 | 26 |
| Lettuce, <i>Lactuca sativa</i> L. | R | — | 119 | 120 |
| Lilac, <i>Syringa</i> sp. | I | — | 37 | 330 |
| Lily, <i>Lilium speciosum</i> Thunb. var. <i>rubrum</i> | R | R | 0 | 44 |
| Lily, regal, <i>Lilium regale</i> Wils. | S | — | 0 | 82 |
| Peach, <i>Prunus</i> sp. var. <i>Elberta</i> | S | — | 0 | 222 |
| Peanut, <i>Arachis hypogaea</i> L. | I | — | 154 | 162 |
| Philadelphus, <i>Philadelphus</i> sp. | R | — | 0 | 104 |
| Plantain, <i>Plantago</i> sp. | R | I | 137 | 224 |
| Plum, <i>Prunus</i> sp. var. <i>Burbank</i> | I | S | 0 | 532 |
| Pokeweed, <i>Phytolacca</i> sp. | I | — | 2 | 146 |
| Purslane, <i>Portulaca oleracea</i> L. | R | — | 57 | 28 |
| Spiraea, <i>Spiraea Vanhouttei</i> Zabel. | I | — | 0 | 312 |
| Strawberry, <i>Fragaria</i> sp. | R | — | 0 | 235 |
| Weigela, <i>Weigela</i> sp. | R | — | 0 | 132 |

* R = resistant; I = intermediate; S = susceptible.

example, develops a mosaic or mottled appearance several days after exposure to HF gas (Fig. 3) but not when exposed to SO₂ gas. *Polygonum* species are marked especially along the margin of the leaf blade when exposed to HF gas, while SO₂ causes interveinous (intercostal) lesions. Variations occurred with both gases. Sometimes HF causes some spotting over the blade, and SO₂ often causes marginal necrosis. However, there is a tendency for predominance of a particular symptom with each gas applied to dicotyledonous species. Monocotyledonous species (gladiolus, iris, lily) frequently show "tip burn" without mottling from exposure to either gas. To identify the causal agent in such cases it is necessary to resort to chemical analyses to determine the amount of fluorine in the tissue. However, all species of plants normally contain fluorine in the tissue. The actual amount



FIGURE 3. Symptoms induced on leaves of sweet corn by fumigation with HF gas for nine days at a concentration of 10 p.p.b. Left to right: varieties Surecross, Golden Cross Bantam, Gold Rush, Spancross, and Marcross. Note two relatively resistant varieties on the left.

TABLE III

SUSCEPTIBILITY AND RESISTANCE OF SPECIES TO FLUORIDE GASES IN RELATION
TO THREE MINERAL CONSTITUENTS (SILICON, CALCIUM,
AND IRON) IN THE TISSUE

| Plant species | Susceptible | Resistant | SiO ₂ , % | CaO, % | Fe, % |
|--|-------------|-----------|----------------------|--------|-------|
| Gladiolus varieties | | | | | |
| Algonquin | X | | 0.14 | 1.64 | 0.013 |
| Snow Princess | X | | 0.07 | 1.51 | 0.011 |
| Leeuwenhorst | X | | 0.06 | 1.62 | 0.012 |
| Aladdin | X | | 0.12 | 1.12 | 0.010 |
| Yellow Emperor | X | | 0.09 | 1.40 | 0.013 |
| Valeria | X | | 0.03 | 1.36 | 0.009 |
| Abu Hassan | X | | 0.26 | — | — |
| Camellia | | X | 0.25 | 1.98 | |
| <i>Chrysanthemum</i> sp. | | X | 2.82 | 2.33 | |
| Cotton | | X | 0.36 | 3.20 | |
| Sweet potato Triumph | X | | 0.54 | 2.26 | |
| Crabgrass [<i>Digitaria sanguinalis</i> (L.) Scop.] | X | | 1.46 | 0.40 | |
| Tobacco | | X | 0.46 | 3.58 | |
| Corn | | | | | |
| Golden Cross Bantam | X | | 2.9 | — | |
| Dixie #17 | X | | 1.1 | — | |
| Rose | | X | 0.70 | 2.94 | |
| Apricot | X | | 0.20 | 1.43 | |
| Prune, Italian | X | | 0.19 | 2.03 | |
| Peach | | | | | |
| Belle of Georgia | X | | 0.31 | 1.81 | |
| White Hall | X | | 0.37 | 1.83 | |
| Hale Haven | X | | 0.28 | 2.14 | |
| <i>Richardia</i> sp. | | | | | |
| Leaves | | X | 3.7 | 6.86 | |
| Stems | | X | 0.72 | 2.94 | |
| Cucumber | | X | 3.9-3.3 | 3.0 | |
| Coleus | X | | 1.2 | 2.2 | |
| <i>Bidens</i> sp. | | X | 3.7 | 4.3 | |

of fluorine absorbed and stored by the plants varies with the species, the age of the tissue, and the locations where the plants grow. While growing in the same vicinity, however, a flowering dogwood stored 40.3 p.p.m. in the leaves while apple leaves contained 7.9 p.p.m. and peach 7.5 p.p.m. *Camellia japonica* L. var. Elegans-Chandler, growing in a greenhouse at

Boyce Thompson Institute, contained 1500 to 2000 p.p.m. in old leaves and variable amounts around 100 p.p.m. in young leaves. Five samples of commercial tea purchased at a store varied in fluorine from 72.7 to 113.5 p.p.m. in the dried leaves. A flowering shrub, *Deutzia* sp., growing on the Institute grounds contained 75 p.p.m. of fluorine while *Weigela* sp., another flowering shrub in the same location, contained only 6 p.p.m. During the course of experimental work with fluorides at Boyce Thompson Institute more than 4500 analyses have been made of some 100 species of plants. All of the samples contained fluorine regardless of where the plants grew. Attempts are now being made to grow some species in fluorine-free nutrients. This should prove whether or not fluorine is an essential element and where the plants may derive their supply.

Of special interest is *Dichapetalum cymosum* Hook., gifblaar, a species native to Africa, known to be poisonous to animals. Two different samples of this plant sent to Boyce Thompson Institute from Africa contained 38.4 and 116 p.p.m. of fluorine in the leaves. According to Peters (9), when the gifblaar leaves are ingested by animals, the monofluoroacetic acid is metabolized to monofluorocitric acid. The monofluorocitric acid is very toxic to animals; for example, 0.2 mg. per kg. of body weight is sufficient to poison the rabbit. It is not known whether fluorine is essential in the metabolism of gifblaar. No other species of plants are known to assimilate and store monofluoroacetic acid.

In attempting to arrive at the reason for susceptibility or resistance of species, analyses were made of 25 species and varieties of plants for the presence of silicon, calcium, and iron. There was no apparent relationship between the presence of these minerals and susceptibility or resistance. The results are tabulated in Table III.

A study was made also of the number and location of stomata in the upper and lower epidermis of leaves of several susceptible and resistant species. Eckerson (1) showed that there was wide variation in the number of stomata in different species of plants and that some species had no stomata in the upper epidermis. Meyer and Anderson (7, p. 143) listed 39 species (including 13 from Eckerson's list) which showed similar variation. Stomatal counts were made at the Boyce Thompson Institute laboratories on 141 species and varieties of plants used in this project. Thirteen of these had been studied previously by Eckerson (1) and Meyer and Anderson (7). In general, the stomatal counts were in good agreement in all studies except for coleus. Eckerson reported no stomata for the upper epidermis of coleus.

The actual stomatal counts per square millimeter of leaf surface are tabulated for 59 species in Tables I and II. There is no apparent relationship between the number of stomata present and the susceptibility of the species to HF or SO₂ gas. For example, Jerusalem cherry which was sus-

ceptible to HF and resistant to SO_2 had about the same number of stomata per unit area (73 upper, 374 lower surface) as Spanish needles (79 upper, 360 lower surface) which was resistant to HF and susceptible to SO_2 . Similar differences in susceptibility were found for a perennial smartweed (23 upper, 99 lower) and tomato (32 upper, 112 lower), yet the leaves of both species had about the same number of stomata per unit area. The average stomatal count for the two sides of leaves of four varieties of gladiolus is as follows: Snow Princess—207, Gold Dust—219, Leeuwenhorst—144, and Bloemfontein—165. Of these four varieties Snow Princess was most susceptible to HF gas, followed closely by Leeuwenhorst and Gold Dust. Bloemfontein was comparatively resistant. Although there are striking differences in relative susceptibility of gladiolus varieties, all are sensitive to HF gas. However, there was no apparent relationship between susceptibility and number of stomata. The number of stomata in 13 varieties of grape ranged from 116 to 268 per square millimeter of lower leaf surface. Two relatively HF resistant varieties, Salvador with 268 stomata per unit area and Muscat of Alexandria with 116, represented the extremes. The most susceptible and intermediate varieties fell somewhere between these two extremes. Four varieties are listed in Table II. As with gladiolus, all varieties of grape are relatively sensitive to HF gas. Also, as with other species, there was no apparent relationship between stomatal number and susceptibility of grape varieties to HF gas.

Susceptibility of leaves varied with the species, the age of the tissue, and the gases involved. For example, tomato plants exposed to HF gas near the threshold concentration showed young, undeveloped leaves with markings (usually tip portions of lobes) while the same species fumigated with SO_2 showed interveinous necrosis and some tip burn on middle-aged leaves. The same gases applied to pines at threshold concentrations caused necrosis of young or undeveloped needles, while mature leaves were not affected with comparatively high concentrations. This variable susceptibility suggests that the constitution of the living matter rather than manner of penetration may determine the susceptibility or resistance of given tissue or species. Leaves of the same age on a given plant were usually not marked alike though they otherwise appeared to be the same. When exposed to higher than threshold concentrations of HF gas, leaves of susceptible species were marked at the margin as well as intercostal areas. A variation of this was found on several species where leaves became speckled with small necrotic areas after the plants were fumigated.

Consideration has been given to the effects of environment on plants prior to the treatments. The condition of the leaves in relation to susceptibility to SO_2 has been published (10, 11, 12). Wilted leaves are more resistant than turgid ones and more susceptible during daylight when stomata are open. The condition of the plant leaves at the time of treatment has an



FIGURE 4. (A) Tomato plants and (B) corresponding enlarged leaves to show variation in degree of susceptibility to HF gas (200 p.p.b. for 6 hrs.) after being conditioned with different amounts of water supply. Left to right for both A and B: (1) plant having an ample water supply throughout; (2) plant having an ample water supply but low fertilizer supply; (3) plant having low water supply throughout the period; (4) water supply low enough to permit plant to wilt much of the time during the period it was being conditioned. All plants had ample water supply at the time they were fumigated.

TABLE IV
COMPARATIVE FLUORINE CONTENT (P.P.M.) IN UPPER FOUR INCHES OF GLADIOLUS
LEAVES* AND IN ADJACENT FOUR INCHES BELOW

| Variety | Control | | Treated** | |
|---------------------|---------|-------|-----------|-------|
| | Upper | Lower | Upper | Lower |
| Rosa van Lima | 54 | 17 | 49 | 21 |
| Snow Princess | 58 | 17 | 87 | 37 |
| Elizabeth the Queen | 89 | 14 | 189 | 28 |
| Commander Koehl | 95 | 22 | 101 | 34 |
| Stoplight | 94 | 20 | 218 | 28 |
| Yellow Emperor | 99 | 22 | 113 | 44 |
| Abu Hassan | 107 | 25 | 206 | 32 |

* Composite sample of third, fourth, and fifth leaves.

** Plants exposed August 17 to 24 to concentrations of HF ranging from 0 to 3 p.p.b.

TABLE V
RELATION BETWEEN RELATIVE LENGTH OF LEAF MARKED AND THE FLUORINE CONTENT
OF THE TIP AND BASAL HALVES OF CORN LEAVES ON PLANTS EXPOSED FOR SEVEN
DAYS TO DIFFERENT CONCENTRATIONS OF HF GAS

| Variety of corn | Percentage length of leaf marked | Fluorine content (p.p.m.) of leaves according to concentration of HF gas and part of leaf analyzed | | | |
|------------------|----------------------------------|--|------------|--------------------|------------|
| | | 2.1 to 3.4 p.p.b. HF | | 13 to 14 p.p.b. HF | |
| | | Tip half | Basal half | Tip half | Basal half |
| Tennessee #10 | 75 | 18 | 6 | 175 | 93 |
| | 50 | 21 | 8 | 156 | 82 |
| | 25 | 12 | 4 | 133 | 23 |
| | <25 | 6 | 5 | 82 | 20 |
| | Av. | 14 | 6 | 136 | 55 |
| Neal's Paymaster | 75 | 28 | 12 | 204 | 47 |
| | 50 | 19 | 8 | 124 | 27 |
| | 25 | 22 | 4 | 146 | 43 |
| | <25 | 35 | 4 | 148 | 24 |
| | Av. | 26 | 7 | 156 | 35 |
| Dixie #17 | 75 | 53 | 27 | 131 | 28 |
| | 50 | 32 | 8 | 130 | 56 |
| | 25 | 62 | 24 | 127 | 44 |
| | <25 | 39 | 13 | 99 | 40 |
| | Av. | 47 | 18 | 122 | 42 |
| Funk #512 | 75 | 33 | 15 | 108 | 50 |
| | 50 | 23 | 16 | 108 | 52 |
| | 25 | 11 | 8 | 76 | 17 |
| | <25 | 7 | 3 | — | — |
| | Av. | 19 | 10 | 97 | 40 |
| Funk #134 | 75 | 17 | 12 | 129 | 71 |
| | 50 | 30 | 14 | 141 | 59 |
| | 25 | 39 | 21 | 160 | 71 |
| | Av. | 28 | 16 | 143 | 67 |

effect upon susceptibility to HF gas. For example, withholding water to cause wilting at intervals for 10 days caused tomato and sweet potato plants to become more resistant to HF gas. Figure 4 shows leaves of tomato plants which were exposed for six hours to 200 p.p.b. of HF gas. The leaf on the left came from a plant grown with an ample water supply; the two on the right with no necrosis came from plants which were conditioned by growing with a low water supply. At the time of fumigation all plants had ample water and the leaves were turgid. In another test pot-grown tomato and field corn were preconditioned in a similar way for three weeks before a seven-day exposure, with ample water supply, to an average daily concentration of 7 p.p.b. HF gas. The fluorine content of leaves after treatment was as follows: for control tomato and corn receiving an ample water

TABLE VI

RELATION BETWEEN THE FLUORINE CONTENT AND RELATIVE AMOUNT OF MOTTLING FOR DIFFERENT PARTS OF DIXIE #17 CORN LEAVES EXPOSED SIX DAYS TO HF GAS

| Lengthwise part of leaf | F content (p.p.m.) and relative amount of mottling† according to concentration of HF (p.p.b.) and crosswise part of leaf analyzed | | | | | |
|----------------------------|--|-------------------------|-------------------|-------------------------|-------------------|-------------------------|
| | 0 | | 2.5 | | 7 | |
| | Marginal strip | Strip next to midrib | Marginal strip | Strip next to midrib | Marginal strip | Strip next to midrib |
| Terminal half | 10* | 11* | 38 ⁺⁺ | 8* | 99 ⁺⁺ | 66 ⁺⁺ |
| | | | 31 ⁺ | 13* | 171 ⁺⁺ | 52 ⁺⁺ |
| Basal half | 10* | 3* | 32 ⁺ | 10* | 93 ⁺⁺ | 45 ⁺⁺ |
| | | | 33 ⁺ | 14* | 77 ⁺⁺ | 21 ⁺⁺ |

† None (*), slight (+), and moderate (++)

supply, 146 and 156 p.p.m. respectively; and for plants receiving a low water supply, 193 and 166 p.p.m. for tomato and corn respectively. Thus preconditioning did not prevent the absorption of fluorine.

Results from experimental fumigation of various species of plants form a basis for diagnosing symptoms in fields around industrial operations. Since the symptoms are similar in some cases and variable in others, it is necessary to consider more than one species of plants. By an examination of several species differences can be evaluated and generally a correct diagnosis can be made. For example, if one finds smartweed marked, the causal agent could be either SO₂ or HF gas. If both corn and smartweed are marked, the diagnosis would be in favor of HF gas. However, if alfalfa and smartweed are marked, the active agent would, in all probability, be SO₂ gas. This latter conclusion is drawn because alfalfa is susceptible to SO₂ gas and very resistant to HF gas. When symptoms are not convincing, it is

possible to resort to chemical analysis for fluorine which builds up in leaves as plants are exposed to HF gas.

When samples of vegetation were collected for fluorine analyses, care was taken to select the parts which by experience were apt to be most revealing. For example, the terminal four inches of gladiolus leaves contained more fluorine than the basal part (Table IV). The leaves of corn varied in the amount of fluorine stored with the location on the stalk and the portion of the leaf analyzed. The terminal portion of the leaf contained

TABLE VII
FLUORINE CONTENT IN DIFFERENT PARTS OF LARGE BONNY BEST TOMATO PLANTS
(24-30 INCHES TALL), ALFALFA, AND CELERY AFTER EXPOSURE TO HF
GAS DURING JULY, 1954

| Part of plant analyzed | Fluorine (p.p.m.) in tissue according to concentration of HF (p.p.b.) in air | | |
|--|--|-------------|--------------|
| | Control | 26 p.p.b. | 80 p.p.b. |
| Tomato exposed 24 hours | | | |
| Leaflets from middle-aged leaves | 13 | 174 | 327 |
| Lower leaves | 10 | 149 | 304 |
| Composite sample of different-aged leaves | 9 | 162 | 176 |
| Upper leaves | 8 | 69 | 109 |
| Top, including young leaves and growing tips | 8 | 26 | 43 |
| Stems | 2 | 5 | 6 |
| Fruit | 2 | 2 | 4 |
| Alfalfa exposed eight days | | | |
| | Control | 1-6 p.p.b. | 1-4 p.p.b. |
| Top, new growth | 7 | 15 | 14 |
| Leaves, new growth | 7 | 20 | 37 |
| Stems, new growth | 1 | 2 | 5 |
| Leaves, old growth | 15 | 512 | 703 |
| Stems, old growth | 3 | 91 | 77 |
| Celery exposed six days | | | |
| | Control | 0-23 p.p.b. | 16-33 p.p.b. |
| Leaflets | 10 | 224 | 905 |
| Stalks | 2 | 6 | 47 |
| Heart | 4 | 5 | 17 |

more than the basal portion (Table V), and the marginal area contained more than the part next to the midrib (Table VI). There was no close correlation between the relative amount of mottling and the fluorine content of corn leaves exposed to HF gas. In general, there was more fluorine in the leaves than in the stems and more in the stems than in the fruit. The results for tomato, alfalfa, and celery appear in Table VII.

ACKNOWLEDGMENTS

The authors acknowledge with thanks the assistance received from various staff members of Boyce Thompson Institute. Mr. R. C. Thompson

designed and built the air scrubbers and special ducts for distributing air uniformly throughout the portable greenhouses. Dr. Radu Mavrodineanu and Mr. Robert R. Coe designed and managed the equipment for dispensing the gas into the fumigation chambers. Mrs. Mary Vogel and Mr. Coe had charge of air analyses to determine the concentration of HF gas in the portable greenhouses and stationary cabinets. Messrs. J. Gwirtsman and Donald Frey analyzed plant tissue for fluorine. Miss Eugenia Keblish studied and counted the stomata in the epidermis of leaves.

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Note

TWO NEW HOSTS OF MATSUCOCCUS BARK SCALE

ALBERT HARTZELL

Before 1946, the only species of coccid of the genus *Matsucoccus* known to occur in Northeastern United States was *Matsucoccus gallicolus* Morr., which was confined chiefly to pitch pine (*Pinus rigida* Mill.) and scrub pine (*Pinus contorta* Dougl.). In Easton, Connecticut in November 1946, George H. Plumb found a number of red pine (*Pinus resinosa* Ait.) heavily infested with a species of *Matsucoccus*. This proved to be a new species later described as *Matsucoccus resinosae* Bean and Godwin.¹ By 1953 a survey of Connecticut showed that this infestation extended over an area of 60 sq. mi. This scale was first reported in New York in 1950. An extensive survey made by the State of New York in 1953 showed an infested area of approximately 40 sq. mi. on Long Island, and scattered spot infestations along the Hutchinson Parkway and in Yonkers, Scarsdale and Tuckahoe, Westchester County, New York.

As a grove of about 100 red pine trees in the Boyce Thompson arboretum was found to be infested with *Matsucoccus resinosae*, a cooperative project between the Forest Service of the United States Department of Agriculture, the New York State Entomologist's Office, the New York State Department of Conservation, and the Boyce Thompson Institute for Plant Research was begun in 1954 to determine the host range. Because of a considerable number of exotic species of pine trees growing in the arboretum the locality afforded an excellent opportunity to determine whether this species of scale is confined to a single species of pine, as was suspected, or was capable of establishing itself on other hosts.

During the growing season of 1954 and 1955 attempts were made to infest twenty-two species of pine in the arboretum with *Matsucoccus resinosae* scale by tying infested twigs of red pine to the outer branches during the months of June 1954 and June and August 1955, when crawlers were present. The infested red pine twigs were allowed to remain appressed to the branches throughout the remainder of the growing season. An examination of these trees in the autumn of 1954 for evidence of living scales of this species was negative. However, when the trees were examined on November 9, 1955, a Japanese red pine (*Pinus densiflora* Sieb. & Zucc.) tree which had been exposed as described above on June 28, 1954 and June 5 and August 4, 1955 was found to be infested with living *Matsucoccus resinosae* scale. Many first stage nymphs were apparently overwintering on

¹ Bean, James L., and Paul A. Godwin. Description and bionomics of a new red pine scale, *Matsucoccus resinosae*. Forest Science 1: 164-176. 1955.

the 1951 twig growth. Also several second instar nymphs were found on the four- and five-year-old twig growth. The infestation on this tree was equal in density to that of the red pine trees in the infested grove in the arboretum. A Chinese pine (*Pinus tabulaeformis* Carr.) also was found to be infested. This tree likewise had been exposed by having red pine twigs infested with *Matsucoccus resinosae* scale tied to its branches on June 15, 1954, and again on June 15 and August 4, 1955, as described previously. Two live nymphs were found on four-year-old twig growth and many exuviae or cast skins were present. No living scales of *Matsucoccus resinosae* were found on any of the other twenty species of pine in the arboretum as listed below:

Pinus aristata Engelm.

P. armandi Franch.

P. ayacahuite Ehrenb. (*P. hamata* Roetzl.)

P. bungeana Zucc.

P. cembra L. var. *sibirica* Loud.

P. flexilis James

P. griffithii McClelland (*P. excelsa* Wall.)

P. heldreichii Christ. var. *leucodermis* (Ant.) Markgraf ex Fitschen

P. koraiensis Sieb. & Zucc.

P. lambertiana Dougl.

P. monticola Lamb.

P. mugo rostrata (Ant.) Hoopes (*P. montana* Mill., *P. uncinata* Ramond)

P. nigra poiretiana (Ant.) Aschers & Graebn. (*P. laricio* Poir. var. *calabrica* Loud.)

P. parviflora Sieb. & Zucc. (*P. pentaphylla* Mayr.)

P. peuce Griseb.

P. ponderosa Laws.

P. rigida Mill.

P. strobus umbraculifera Carr.

P. sylvestris L. var. *rigensis* Loud.

P. thunbergii Parl.

The writer is especially indebted to Mr. Donald P. Connola of the New York State Entomologist's Office, Albany, New York, and to Mr. Thomas McIntyre, Entomologist of the United States Department of Agriculture, Forest Service, New Haven, Connecticut, for assistance in the identification of *Matsucoccus resinosae* on these two host plants.

The existence of more than one host of *Matsucoccus resinosae* adds to the complexity in making surveys and in the control of this species of scale.

THE ROLE OF AUXIN IN PLANT FLOWERING. IV. A NEW UNIDENTIFIED NATURALLY OCCURRING INDOLE HORMONE IN NORMAL AND GAMMA IRRADIATED MARYLAND MAMMOTH TOBACCO

A. J. VLITOS, WERNER MEUDT, AND R. BEIMLER

SUMMARY

Short-day tobacco (*Nicotiana tabacum* L. var. Maryland Mammoth) flowered under long daylengths when exposed to gamma rays at dosages of 50, 150, 165, 300, and 430 roentgens per day for 60 days. Flowering occurred irrespective of daylength in over 75 per cent of the progeny from parents which had been subjected to gamma rays. When the offspring of parents which had been treated with 150 roentgens per day for 60 days were photoinduced by successive short-day cycles, they flowered sooner than normal Maryland Mammoth tobacco.

A study was made of the relationship of the naturally occurring indole compounds to the induction of flowering in gamma irradiated plants. 3-Indoleacetic acid was not detected in leaves, stems, roots, or apices of normal, of gamma irradiated, or of offspring of gamma irradiated parents when ethanol extracts of 500 g. or more of these tissues were chromatographed and eluates from the chromatographs tested for biological activity in pea (*Pisum sativum* L. var. Alaska) curvature or tomato (*Lycopersicon esculentum* L. var. Bonny Best) petiole epinasty tests. Employing the split pea curvature, tomato petiole epinasty tests, and *Avena* curvature and straight-growth tests as criteria of plant hormone activity for cell elongation, an unidentified indole component was found to be the predominant auxin in Maryland Mammoth tobacco.

Ultraviolet absorption spectra, colorimetric data, and chemical reactivity with acids and bases indicated that the unidentified substance contains an indole ring, is stable to treatment with alkalis, and labile to acid treatment. The material was chromatographed in 17 different solvents. Biologically, it was active in inducing curvatures of split-pea and *Avena* sections and epinasty of tomato petioles. It was found in leaf and apical tissues but not in root or stem tissues of both normal and gamma irradiated Maryland Mammoth tobacco plants. It does not appear to be 3-indoleacetic acid, the ethyl ester of 3-indoleacetic acid, or 3-indoleacetonitrile, the three most common naturally occurring plant hormones. Although there is a possibility that the unidentified substance may be a mixture, ultraviolet absorption spectra, colorimetric data, and chemical reactivity with acids and bases indicated that it is a single compound containing an indole ring. These results are discussed in the light of current theories regarding plant growth hormones and photoperiodism.

INTRODUCTION

In the current concepts regarding the role of natural growth hormones in the flowering process it is suggested that high endogenous levels of hormone in short day plants delay or completely suppress flowering. A reduction in the level of 3-indoleacetic acid has been regarded to be favorable for the formation of floral primordia (1, 2).

Previous papers in this series have been concerned with the quantitative determination of indole compounds in photoinduced, short-day plants.

It was reported (9) that a substantial increase occurs in the level of 3-indoleacetic acid and 3-indolepyruvic acid after photoinduction of short-day soybean (*Glycine max* Merr. var. Biloxi).

Reports in the literature suggest that 3-indoleacetic acid is destroyed *in vivo* and *in vitro* by ionizing radiations (3, 7). It seemed logical to assay the influence of gamma rays on the flowering behavior of a short-day plant, such as Maryland Mammoth tobacco and to study the relationship, if any, between flowering and the destruction or reduction of 3-indoleacetic acid or other growth hormones by gamma rays.

GAMMA RAY EXPERIMENTS

During the summer of 1954 one-month-old seedlings of Maryland Mammoth tobacco were planted in the gamma field of the Brookhaven National Laboratory¹ at Upton, Long Island, New York. The seedlings had been grown in the greenhouse under long photoperiods, and were in a vegetative state at the time of transplanting. In the gamma field, fifteen seedlings were exposed for 60 days to each of five dosages of radiation: 50, 150, 165, 300, and 430 roentgens per day, obtained by placing the plants 29, 17, 16, 12, and 10 meters, respectively, from the radioactive cobalt source. The prevailing daylengths during the experiments were long enough ($14\frac{1}{2}$ to $15\frac{1}{2}$ hours) to maintain control plants in a vegetative condition, since the critical photoperiod for flowering of Maryland Mammoth tobacco is between $8\frac{1}{2}$ to $9\frac{1}{2}$ hours. After 60 days of continuous exposure to the various dosages of gamma radiation, examinations were made for the presence of macroscopic flowers. The morphological appearance of typical plants which had been subjected to the various dosages of gamma rays for a total of 60 days is presented in Figure 1.

Macroscopic flowers were formed on all plants which had been exposed to dosages of 50 roentgens per day for 60 days. Those plants which received dosages of radiation exceeding 50 roentgens per day exhibited various types and degrees of radiation damage, ranging from "string-leaf" patterns to complete suppression of apical dominance. Flowers had not formed on these plants at the end of the 60-day exposure period. However, when the plants were removed from the gamma field, transferred to the greenhouse, and their growth continued under 16-hour photoperiods, supplemental light being provided by 500-watt Mazda incandescent lamps, they produced normal leaves, resumed stem elongation, and flowered within three weeks. Control plants of comparable age which had been grown under similar, long photoperiods, but which had not been exposed to gamma-rays, remained vegetative.

¹ The authors are indebted to Dr. Seymour Shapiro of the Brookhaven National Laboratory for his cooperation and many suggestions during the course of the experiments.

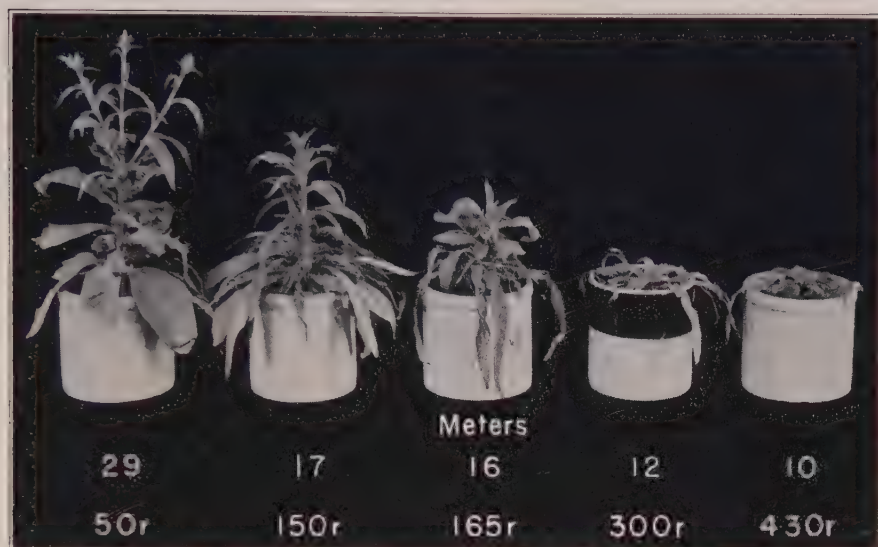


FIGURE 1. Maryland Mammoth tobacco plants after 60 days of exposure to gamma rays at the indicated meters from source. Left to right: plants received 50, 150, 165, 300, and 430 roentgens per day. These plants subsequently resumed normal growth and flowered under long daylengths.

GROWTH AND FLOWERING CHARACTERISTICS OF THE OFFSPRING OF GAMMA RAY TREATED PLANTS

Seed were collected at random from the gamma irradiated plants, all of which had flowered and produced seed pods after open pollinations had taken place under long photoperiods. The seed were germinated in a composted sod soil in the greenhouse, and the seedlings grown under 16-hour photoperiods in the greenhouse. Control seed, obtained from parents which had been isolated from gamma irradiated plants, were germinated and the seedlings grown under similar 16-hour photoperiods. After two months under these nonphotoinductive cycles, 150 plants representing the offspring of gamma irradiated and normal parents were transferred during the summer of 1955 to the field and planted in a randomized block design. The plants continued to grow under long photoperiods ($14\frac{1}{2}$ to $15\frac{1}{2}$ hours) in the field. The appearance of macroscopic flowers was recorded at intervals throughout the growing season. Results are given in Table I.

Control plants remained vegetative throughout the growing season whereas 43 per cent and 28 per cent of the progeny of parents which had received 150 and 165 roentgens per day for 60 days respectively had formed macroscopic flowers. Macroscopic flowers had formed after 90 days in 5 per cent of the progeny of parents which had received 300 roentgens per day while none were formed on progeny of plants which had been subjected

to 430 roentgens per day for 60 days. Flowering, where it occurred, took place in plants never exposed to short-days.

The number of photoinductive cycles (successive 18-hour dark periods) that are necessary for floral induction of progeny of plants which received 150 roentgens per day for 60 days was determined. Plants which had been grown for 45 days at 72° to 75° temperatures and under 16-hour light

TABLE I
FLOWERING OF OFFSPRING OF GAMMA IRRADIATED AND NORMAL PLANTS OF MARYLAND
MAMMOTH TOBACCO IN THE FIELD UNDER LONG-DAY CYCLES
(14½ TO 15½ HOURS)

| Daily dosage of gamma radiation given parent plants (roentgens) | Percentage of 30 plants with macroscopic flowers after (days) | | | | | |
|---|--|----|----|----|----|----|
| | 48 | 55 | 64 | 74 | 76 | 90 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 150 | 8 | 14 | 25 | 33 | 43 | 43 |
| 165 | 14 | 14 | 16 | 26 | 28 | 28 |
| 300 | 5 | 5 | 5 | 5 | 5 | 5 |
| 430 | 0 | 0 | 0 | 0 | 0 | 0 |

periods in constant temperature-light rooms equipped with mercury-vapor arc lamps were transferred to days with 18-hour dark periods for 0, 7, 14, 21, and 28 days. At the termination of the photoinductive treatment the plants were transferred back to 8-hour dark periods. Macroscopic flowers formed on 40 per cent of the progeny of irradiated plants receiving 21 cycles and on 100 per cent of those receiving 28 photoinductive cycles whereas only 20 per cent of comparable control plants flowered after 21 and 28 photoinductive cycles.

EFFECT OF GAMMA RADIATION ON THE INDOLE COMPOUNDS IN PARENTS AND PROGENY

The following series of experiments were carried out to determine whether the exposure to gamma radiation had affected the ability to synthesize 3-indoleacetic acid and related compounds and, if so, whether such changes were correlated with changes in flowering habit. Leaves, stems, apices, and roots of normal, gamma irradiated, and progeny of irradiated parents were extracted with absolute ethanol and the extracts chromatographed under conditions which are described in detail below. Biological tests were carried out with eluates from paper chromatograms in an effort to determine whether the compounds possessed physiological activity.

EXTRACTION OF TISSUE

The extraction of tissues of normal, gamma irradiated, and offspring of gamma irradiated parents was carried out in a manner described previously

(8). Approximately 300 g. each of leaf, stem, root, and apical tissue were harvested, frozen rapidly, and homogenized with absolute ethanol in a Virtis "45" homogenizer. The homogenates were extracted with absolute ethanol at -10°C . for 12 to 14 hours, the extracts centrifuged, the residues washed three times with fresh, absolute ethanol, and the washings combined with the original supernatant. The ethanol was evaporated off by an air stream, leaving an aqueous fraction to which was added a few drops of chloroform or toluene. The aqueous fraction was adjusted to pH 3.5 to 4.0 with 0.1*N* H_3PO_4 , extracted with ethyl ether, and the ether fractions concentrated, by evaporation, to 2.5 to 2.0 ml.

PAPER CHROMATOGRAPHY OF EXTRACTS

The system of paper chromatography which was employed to detect the indole compounds in the extracts was the same as that which has been described in earlier papers (8, 9). Biological tests have been added to the technique to detect physiological activity of compounds in the chromatographic spots. Those areas on an unsprayed chromatogram, corresponding to areas in which an indole compound was detected colorimetrically with *p*-dimethylaminobenzaldehyde, were cut out of the chromatogram, eluted with distilled water or dilute sodium bicarbonate solution, and the eluates then tested for cell elongation activity in the split-pea curvature test.

The *R_f* values in 17 different solvents for the only indole substance (unidentified) which was detected in extracts of leaves and apices of normal, gamma irradiated, and progeny plants are listed in Table II, together with *R_f* values for the most common growth hormones found in plant tissues (3-indoleacetic acid, 3-indoleacetonitrile, and the ethyl ester of 3-indoleacetic acid). The *R_f* values for skatole and tryptophan, two common naturally occurring indole derivatives, are also included. The values represented in Table II are those obtained for at least five replicate chromatograms run at different times in each solvent.

BIOLOGICAL ACTIVITY

The unidentified indole component, which has been detected consistently in 25 or more individual extractions of apices and leaves, of normal and gamma irradiated Maryland Mammoth tobacco, has been characterized partially by biological and chemical methods.

In assays of biological activity, water eluates from chromatograms which were developed in 2-propanol:ammonia:water (80:5:15) were active in inducing curvature of split-pea sections (Fig. 2). Standard solutions of 3-indoleacetonitrile were inactive in this test in the range of 1 to 1,000 p.p.m., while 3-indoleacetic and the ethyl ester of 3-indoleacetic acid were active in the range of 1 to 1,000 p.p.m. The eluates of chromatograms containing the unidentified indole substance of Maryland Mammoth

TABLE II
R_F VALUES IN 17 SOLVENTS FOR THE UNIDENTIFIED INDOLE COMPONENT IN NORMAL AND GAMMA IRRADIATED MARYLAND MAMMOTH TOBACCO AND FOR VARIOUS NATURALLY OCCURRING INDOLE DERIVATIVES

| Compound | Solvent (R _f value*) | | | | | | | | | | | | | | | | |
|---|---------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
| Unidentified indole component of tobacco | .98 | .98 | .98 | .95 | .98 | .97 | .95 | .99 | .98 | 1.0 | .98 | .98 | .98 | .99 | .67 | .61 | .82 |
| 3-Indoleacetic acid (Eastman Kodak) | .57 | .52 | .85 | .84 | .50 | .78 | .35 | .87 | .98 | .83 | .60 | .60 | .85 | .57 | .80 | .65 | .98 |
| 3-Indoleacetonitrile (Carbide & Carbon Chem. Co.) | .98 | .98 | .85 | .75 | .98 | .95 | .99 | — | .98 | — | — | — | .98 | — | .40 | .65 | .60 |
| Ethyl ester of 3-indoleacetic acid (Carbide & Carbon Chem. Co.) | — | .98 | — | — | .98 | .97 | .96 | .99 | .98 | 1.0 | .98 | .98 | — | .99 | .69 | .78 | .87 |
| Skatole (Eastman Kodak) | — | .98 | — | — | .98 | .86 | .99 | .97 | .98 | — | — | — | .97 | — | — | — | — |
| Tryptophan (Eastman Kodak) | .45 | .45 | — | — | — | — | .45 | — | .51 | — | — | — | .47 | — | .45 | — | — |

* Colors developed with *p*-dimethylaminobenzaldehyde.

Key to solvents:

1. 2-Propanol:ammonia:water (80:1:1)
2. 2-Propanol:ammonia:water (80:5:15)
3. 2-Propanol:ammonia:water (45:5:50)
4. 2-Propanol:ammonia:water (10:5:85)
5. 2-Propanol:ammonia:water (10:1:1)
6. 70% Ethanol
7. 1-Butanol (chamber equilibrated with 5.0% ammonia)
8. 1-Butanol:water:pyridine (1:1:1)
9. 1-Butanol:water:acetic acid (60:25:15)
10. 2-Methyl-2-propanol:2-butanone:water:diethylamine (40:40:20:4)
11. Phenol:water (4:1)
12. Phenol:ammonia (100 ml. — 0.5 ml. chamber equilibrated with ammonia)
13. 1-Propanol:ammonia:water (60:30:10)
14. Ethanol:water:ammonia (90:5:5)
15. Water
16. Water (chamber equilibrated with hydrochloric acid)
17. Water (chamber equilibrated with ammonia)

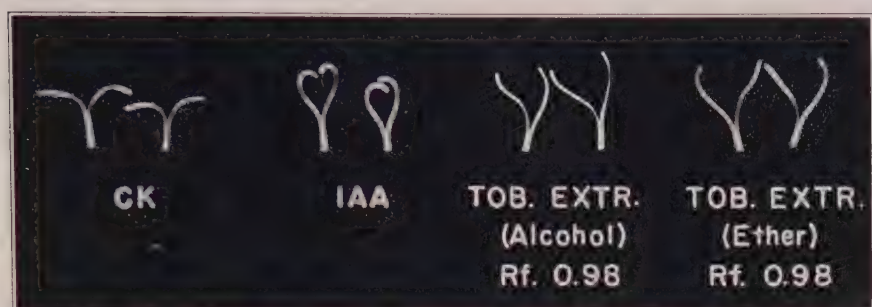


FIGURE 2. Split-pea curvature sections showing from left to right: controls, curvature in 10 mg./l. of IAA, and curvature in eluates of tobacco extracts taken at Rf 0.98 from Whatman No. 1 papers which were developed in 2-propanol:ammonia:water (80:5:15). 8.0 mg. of tobacco tissue were represented in the eluate from the chromatogram.

tobacco were active in the split-pea test when as little as 8 mg. of tissue was represented in the chromatographic spot. When extracts representing 150 to 200 g. of tobacco tissue were dialyzed and the dialysate concentrated to 2 ml., taken up in lanolin and applied to petioles of tomato seedlings, a strong epinastic response was obtained within four hours (Fig. 3).

Through the courtesy of Dr. K. V. Thimann and Dr. Bruce Stowe at Harvard University the unknown was assayed for activity in the *Avena* curvature and straight-growth tests. Excellent curvature was obtained with the concentrated extract from 165 g. of leaf tissue, equivalent to that obtained with 10 micrograms per ml. of 3-indoleacetic acid. The length of curved zones of *Avena* coleoptiles in two tests averaged 8.9 and 7.8

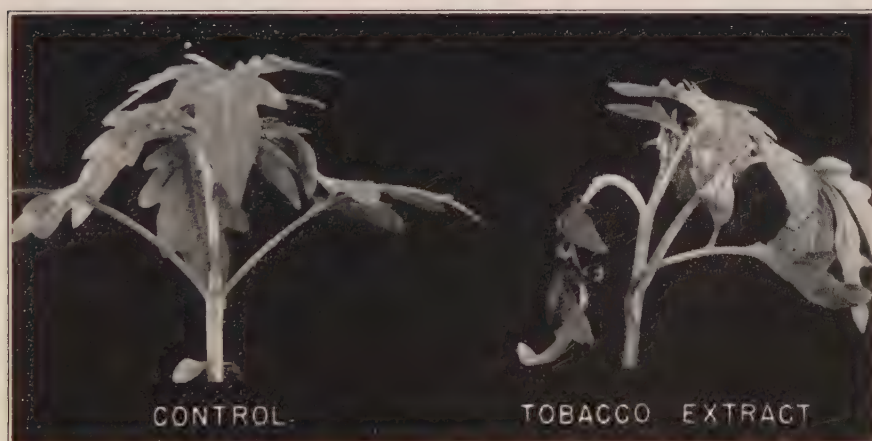


FIGURE 3. Epinasty of tomato petioles (on the right) induced with lanolin preparations of extracts containing the indole hormone of Maryland Mammoth tobacco. Lanolin treated control is on the left.

mm. for the unknown, while the corresponding lengths in coleoptiles treated with 3-indoleacetic acid were 10.0 and 9.7 mm., respectively. Comparable results from the *Avena* section straight-growth assay were obtained with eluates from the upper third of chromatograms containing the unidentified indole hormone, the middle and lower thirds of the paper showing no auxin activity.

CHEMICAL AND PHYSICAL PROPERTIES

The unidentified, physiologically-active substance has been partially characterized chemically by ultraviolet absorption spectra, acid and alkaline treatment, and colorimetric tests. Positive color tests for the indole ring were obtained in reactions with *p*-dimethylaminobenzaldehyde, Salkowski reagent, and the Hopkins-Cole test (5, p. 52) (Table III).

The positive color reactions which were obtained with eluates of paper chromatograms containing the unidentified component suggested that an

TABLE III

COMPARATIVE COLORIMETRIC REACTIONS OF THE INDOLE-CONTAINING COMPONENT OF MARYLAND MAMMOTH TOBACCO WITH OTHER KNOWN INDOLE COMPOUNDS

| Compound | <i>p</i> -Dimethylamino-benzaldehyde | Salkowski | Hopkins-Cole |
|--|--------------------------------------|-----------|--------------|
| Unidentified indole hormone of tobacco | Purple-blue | Brown-red | Purple-blue |
| 3-Indoleacetic acid | Purple | Pink | Purple-blue |
| 3-Indoleacetonitrile | Red-pink | Blue | Pink |
| Ethyl ester of 3-indoleacetic acid | Blue | Purple | Purple-blue |
| Skatole | Blue | Yellow | Red |
| Tryptophan | Pink | Yellow | Red |

indole ring was present in the molecule. The similarities in color reactions between the ethyl ester of 3-indoleacetic acid and the unknown, particularly with *p*-dimethylaminobenzaldehyde and with Salkowski reagent, coupled with similarities in their *R_f* values in most of the solvents which were tried, suggested that the unidentified hormone might in fact be the ethyl ester of 3-indoleacetic acid. Therefore, acid and alkaline treatments were carried out with authentic samples of the ethyl ester of 3-indoleacetic acid and with eluates of chromatograms containing the unknown. For acid hydrolyses 3 ml. of an aqueous eluate from paper chromatograms containing the equivalent of 300 g. of normal Maryland Mammoth tobacco leaf tissue were refluxed for two hours in 7 ml. of 6*N* HCl. The pH of the hydrolysate was adjusted to 6.7 by the addition of 0.5*N* NaOH. The hydrolysate was evaporated to dryness and extracted with 50 ml. of ethyl ether. The ether extracts were concentrated to 2.5 ml., 20 microliters of the extracts spotted on Whatman No. 1 paper and chromatographed in three solvent systems (70% ethanol, H₂O in a chamber equilibrated with HCl, and H₂O

in a chamber equilibrated with NH_4OH). Authentic samples of the ethyl ester of 3-indoleacetic acid (Carbide and Carbon Chemicals Co.) were hydrolyzed and chromatographed under similar conditions. Five micrograms of the ethyl ester of 3-indoleacetic acid were represented on the chromatogram.

Alkaline hydrolyses of extracts containing the unidentified indole substance were carried out as follows: 3 ml. of eluates containing the equivalent of 300 g. of Maryland Mammoth tobacco tissue were refluxed for two hours in 7 ml. of a 3.0 per cent NaOH solution. Saponification of a 20-milligram sample of ethyl ester of 3-indoleacetic acid was carried out in a similar manner. The reaction mixtures were adjusted to pH 7 after refluxing and were chromatographed. In another set of experiments the eluate containing the unidentified indole component was allowed to stand for 24 hours at room temperature with 10 ml. of 14.8*N* NH_4OH . An authentic sample of the ethyl ester of 3-indoleacetic acid was subjected to similar treatment. Twenty-microliter aliquots of each reaction mixture were chromatographed and developed in 2-propanol:ammonia:water (10:1:1), 70 per cent ethanol, and ethanol:ammonia:water (95:5:5).

RESULTS OF HYDROLYSES

It was found that after acid hydrolysis the ethyl ester of 3-indoleacetic acid yielded 3-indoleacetic acid while the unidentified indole component could not be detected with Ehrlich's reagent, on the chromatograms, suggesting that the indole ring was destroyed or that it was not taken up in ether at pH 6.7.

After alkaline hydrolysis with 3.0 per cent NaOH the ethyl ester of 3-indoleacetic acid yielded 3-indoleacetic acid and a trace of unreacted ester, while the unknown indole component remained unchanged. After ammonolysis in 14.8*N* NH_4OH the ethyl ester of 3-indoleacetic acid yielded 3-indoleacetamide and 3-indoleacetic acid while the unknown did not react.

ULTRAVIOLET ABSORPTION SPECTRA

Ultraviolet absorption spectra were obtained for several known indole derivatives, employing a Beckman Model DU spectrophotometer. These were compared with spectra obtained from 2-propanol eluates of paper chromatograms containing the unidentified hormone. Each eluate from a paper chromatogram contained the quantity of hormone present in 150 g. of leaf tissue of Maryland Mammoth tobacco.

The ultraviolet spectra for indole, skatole, 3-indoleacetonitrile, 3-indoleacetic acid and the unknown did not differ from each other appreciably, agreeing with the work of Grammaticakis (4). A typical ultraviolet absorption spectrum for the ethyl ester of 3-indoleacetic acid and for the tobacco hormone is reproduced in Figure 4. Maximum absorption bands,

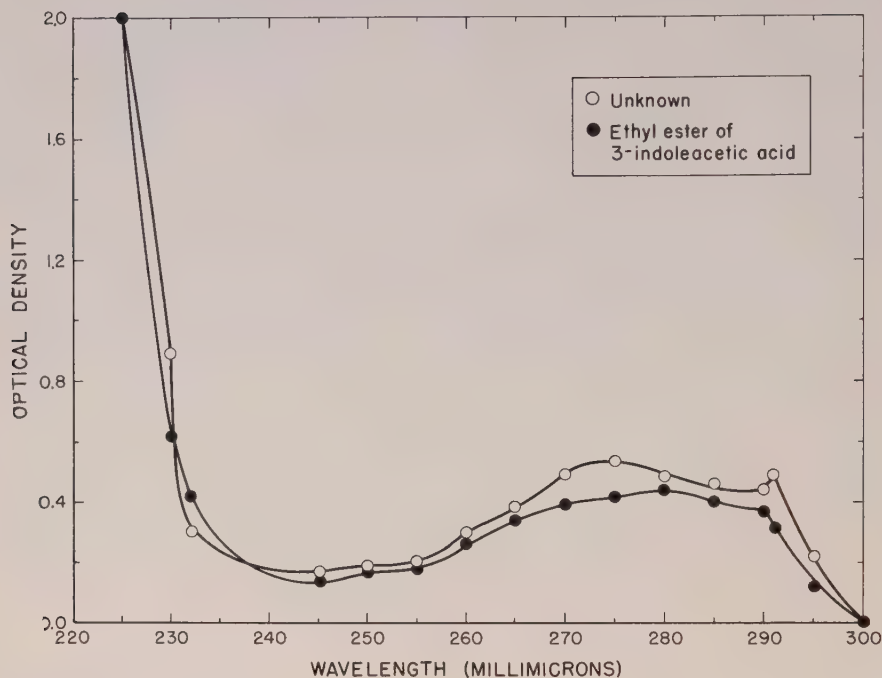


FIGURE 4. Ultraviolet absorption spectra for the ethyl ester of 3-indoleacetic acid ($10^{-4}M$) and for 2-propanol eluates of paper chromatograms containing the unidentified indole hormone in 150 g. of leaf tissue of Maryland Mammoth tobacco.

in millimicrons, for the various indole derivatives which were examined were as follows: indole 280, ethyl ester of 3-indoleacetic acid 280 and 282, skatole 285, 3-indoleacetonitrile 280, 3-indoleacetic acid 280, and the unknown 275. The ultraviolet absorption spectra coupled with the colorimetric data and solubility characteristics suggest that the unidentified hormone of Maryland Mammoth tobacco contains an indole ring.

DISCUSSION

Although gamma irradiation of Maryland Mammoth tobacco results in a change in the flowering behavior of this normally short-day plant, the induction of flowering with gamma radiation cannot be attributed to the destruction of endogenous 3-indoleacetic acid. 3-Indoleacetic acid was not detected in the leaves, stems, roots, or apices of normal plants or in similar parts of gamma irradiated plants or in similar parts of the progeny of gamma irradiated parents. The predominant naturally occurring plant growth hormone in the leaves and apices of Maryland Mammoth tobacco is an unidentified indole component which, on the basis of chemical and biological tests, does not appear to be an ester of 3-indoleacetic acid or 3-

indoleacetonitrile. The unidentified substance has a maximum absorption in ultraviolet at $275\text{ m}\mu$, is stable to alkaline hydrolysis and ammonolysis, is labile when refluxed with acid, and forms positive color complexes with *p*-dimethylaminobenzaldehyde, Salkowski and Hopkins-Cole reagents.

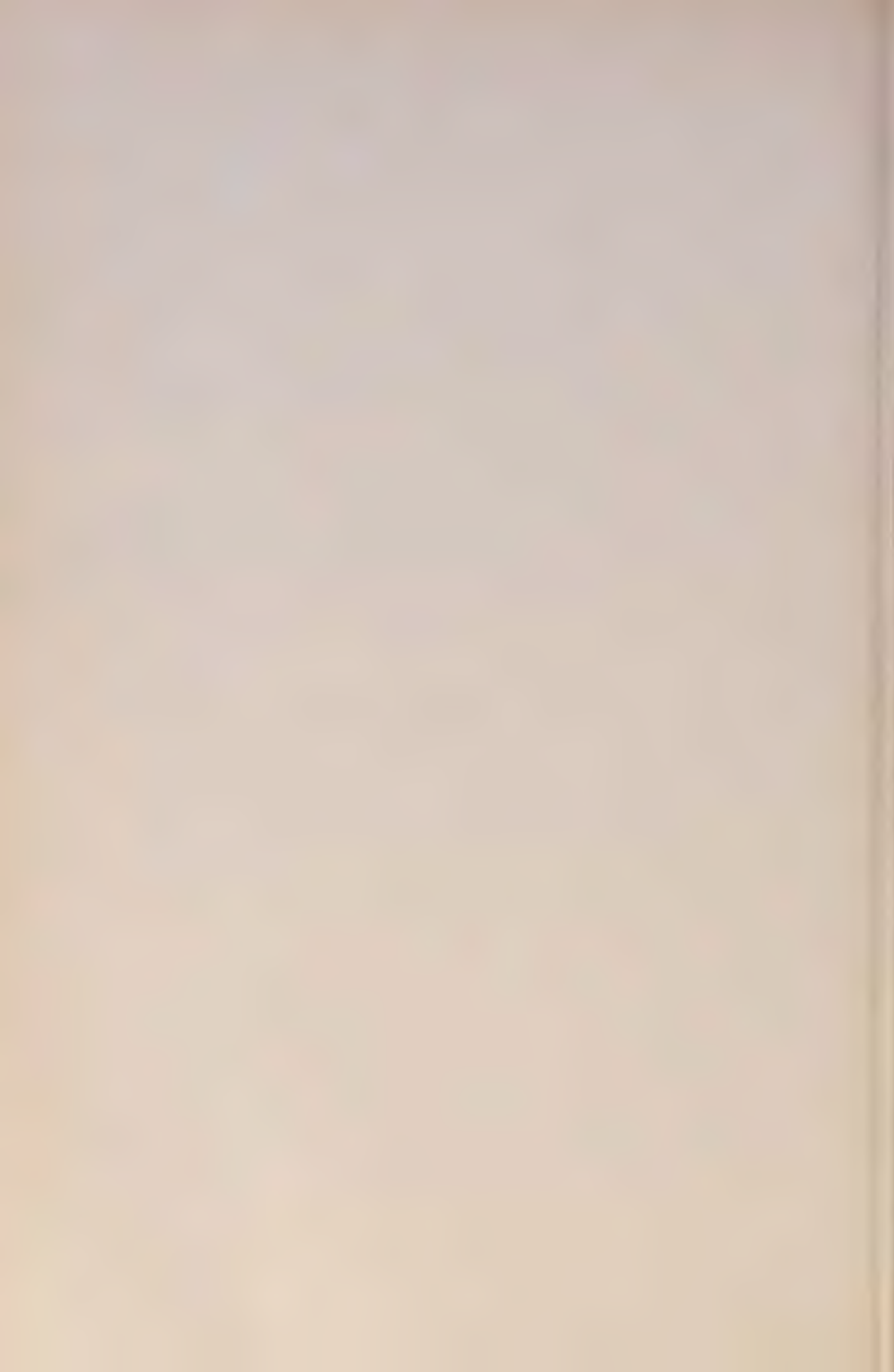
Biologically, the substance has been shown to be active in the split-pea curvature test, *Avena* straight growth and curvature tests, and in the induction of epinasty of tomato petioles. Attempts are being made to isolate sufficient quantities of the material for elemental analyses and for other analytical tests.

The relation of the levels of the naturally occurring indole hormone of Maryland Mammoth tobacco to the induction of flowering with gamma rays has not been defined. Quantitative levels of the hormone can be determined when the compound is isolated, identified, and synthetic samples prepared. It will then be possible to prepare standard calibration curves with authentic samples.

A critical survey of other species of higher plants may reveal that the regulation of growth is not the exclusive function of any single indole compound. This concept is strengthened by the predominance of an indole component, other than 3-indoleacetic acid or one of its esters, in the tissues of Maryland Mammoth tobacco, and by the recent report of Luckwill and Powell indicating the absence of 3-indoleacetic acid in the apple (6).

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PLANT GROWTH-REGULATING EFFECT OF 1,1-DIFLUOROETHYLENE (GENETRON 150) APPLIED TO PLANTS

P. W. ZIMMERMAN AND A. E. HITCHCOCK

SUMMARY

Ethylene gas is a natural product of growing plants and ripening fruit. It is physiologically active when applied to plants and induces hormone-like responses. When the ethylene molecule is substituted with fluorine to make 1,1-difluoroethylene (Genetron 150), it retains physiological activity and regulates plant growth similar to ethylene gas.

The physiological activity of unsaturated hydrocarbon gases applied to plants has been known since 1900 when Neljubow (4) studied the effects of "laboratory air" (manufactured illuminating gas). Ethylene proved to be the active constituent. It has been the subject of many studies (1) since that time and was eventually shown to be a natural product of growing plants and especially of fruit (2, 3, 5).

The purpose of this short paper is to report that the ethylene molecule ($\text{CH}_2=\text{CH}_2$) can be substituted with F_2 to make 1,1-difluoroethylene ($\text{CF}_2=\text{CH}_2$) and still retain physiological activity. Many such substitutions have been made¹ but only 1,1-difluoroethylene (Genetron 150) has been tested.

Genetron 150 was supplied for experimental purposes by Dr. C. Woolf of General Chemical Division, Allied Chemical & Dye Corporation. Like ethylene it is gaseous and is stored in iron cylinders. The plants were exposed to measured amounts of the gas in large glass cases and in glass bell jars. The time of exposure varied from 24 to 96 hours. The degree of response of tomato plants was indicated by the change of angle between the upper side of the petiole of the leaf and the stem of the plant. The change in the position of the leaf is illustrated in Figure 1.

After it was found that Genetron 150 caused epinasty of tomato leaves in a range of concentrations from 500 to 2000 p.p.m. of air, a comparison of activity was made with ethylene gas. Approximately equal degrees of response were induced with 10 p.p.m. of ethylene and 2000 p.p.m. of Genetron 150. The results for one test are given in Table I. There was no apparent difference in the two sets of treated plants. Also Figure 1 shows the similarity of response induced by ethylene and Genetron 150. The concentration of Genetron 150 in this test was, however, 200 times greater than that of ethylene.

Petioles of 12 tomato plants were measured with a protractor at the

¹ Personal communication from Dr. Woolf, February 16, 1956.

TABLE I

COMPARATIVE ACTIVITY OF GENETRON 150 AND ETHYLENE GAS AS INDICATED BY THE CHANGE IN THE DEGREE OF EPINASTIC RESPONSE OF TOMATO PLANTS. THE CHANGE IN ANGLES ON FOUR LEAVES ON EACH SET OF FOUR PLANTS WAS MEASURED BY MEANS OF A PROTRACTOR

| Plant No. | Degree of angles 72 hours after treatment | | | | | | | | | | | |
|--------------------|---|----|----|----|----------------------|-----|-----|-----|--------------------|-----|-----|-----|
| | Control | | | | Genetron 2000 p.p.m. | | | | Ethylene 10 p.p.m. | | | |
| | Leaf number | | | | Leaf number | | | | Leaf number | | | |
| | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| 1 | 50 | 50 | 60 | 60 | 135 | 120 | 145 | 145 | 125 | 130 | 150 | 140 |
| 2 | 60 | 65 | 95 | 70 | 130 | 140 | 140 | 125 | 125 | 130 | 140 | 135 |
| 3 | 60 | 40 | 55 | 70 | 130 | 135 | 135 | 130 | 125 | 130 | 145 | 140 |
| 4 | 65 | 65 | 75 | 85 | 140 | 145 | 145 | 135 | 130 | 125 | 140 | 145 |
| Av. | 59 | 55 | 71 | 71 | 134 | 135 | 141 | 134 | 126 | 129 | 144 | 140 |
| Av. for all angles | 64 | | | | 136 | | | | 135 | | | |

beginning of the experiment, and then 6 of the plants were exposed to 1000 p.p.m. of Genetron 150. All angles were remeasured after 24 and 48 hours. The average for 24 angles of the control plants changed from 46.1° to 56.2°; for the same number of treated plants the average changed from 60° to 108.4°. Most of the increase in angles of treated plants occurred during the first 24 hours.

A large number of plants which responded to treatment with ethylene were listed by Crocker *et al.* (1). The species varied from marked epinasty to slight or no response. Similar results were obtained when 22 species of plants were treated with Genetron 150 (1000 p.p.m.). Tomato (*Lycopersicon*



FIGURE 1. Tomato plants showing epinastic response after 48 hours' exposure. Left, control; middle, exposed to 200 p.p.m. of Genetron 150; right, exposed to 10 p.p.m. of ethylene gas.

persicon esculentum Mill.), buckwheat (*Fagopyrum esculentum* Moench.), perennial smartweed (*Polygonum* sp.), Hubam clover (*Melilotus* sp.), and periwinkle (*Vinca* sp.) made pronounced responses. Peppermint (*Mentha piperita* L.), stevia (*Piqueria trinervia* Cav.), and cotton (*Gossypium hirsutum* L.) showed slight epinasty while tobacco (*Nicotiana tabacum* L.), *Coleus blumei* Benth., orange (*Citrus* sp. var. King), *Begonia* sp., Concord grape (*Vitis* sp.), California privet (*Ligustrum ovalifolium* Hassk.), and geranium (*Pelargonium* sp.) did not show epinasty of leaves. The young leaflets of rose (*Rosa* sp.) curled downward. The petioles and peduncles of *Nasturtium* sp. curled irregularly. The sweet potato (*Ipomoea batatas* Lam.) petioles curled, and the young leaf blades curled downward. There was a tendency of the older leaves to turn yellow and absciss after 96 hours of treatment. The latter symptoms are also characteristic for ethylene gas effects.

From the results so far observed, it appears that ethylene gas and the fluorine-substituted ethylene (1,1-difluoroethylene) induce similar effects on plants. This raises the question of whether numerous other substitutions could be made in the ethylene molecule without causing loss of physiological activity when applied to plants. Further tests will be made as soon as other forms are available.

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Note

PAPER CHROMATOGRAPHY OF AROMATIC ALDEHYDES
AS THEIR SEMICARBAZONES

EDWARD A. PRILL AND DOROTHY FISHER

The aldehydes and ketones are such important constituents of plants that methods are needed for separating and identifying them expediently. Paper chromatography would appear to be a very promising method since highly colored compounds can be secured from carbonyl compounds by developing papers with 2,4-dinitrophenylhydrazine.

Free aldehydes such as vanillin, ethyl vanillin (3-ethoxy-4-hydroxy-benzaldehyde) and piperonal were separated and identified by converting them to 2,4-dinitrophenylhydrazones by Gailey (2). The method was refined later as a micro method for determination of vanillin and ethyl vanillin. Unfortunately this method is not reliable for all aldehydes because the more volatile members may be lost from the papers and others may be oxidized during the long period required from solvent separation.

In order to avoid these losses of aldehydes, studies were undertaken on methods of converting the aldehydes to 2,4-dinitrophenylhydrazones and separating them by various solvents. When the methods of Rice *et al.* (3) were applied to aromatic aldehydes they proved inadequate because the hydrazones would not migrate on paper. Recently several groups of investigators (1, 4, 5) have also pointed out the shortcomings of this and similar procedures. Each of these groups described new solvent systems which they reported gave good separations of the 2,4-dinitrophenylhydrazones of the carbonyl compounds tested (mainly aliphatic).

A new method has been devised for the separation of aromatic aldehydes following their conversion to semicarbazones.

For the present tests, the semicarbazones of the aldehydes indicated in Table I were prepared by standard methods and recrystallized until they had the correct melting points. The ascending chromatographic procedure was essentially that used by Way and Gailey (6) for the free aldehydes. In addition to the solvent system used by Way and Gailey, two other solutions were tried. The three different solvent systems were the following:

A. *n*-Butanol saturated with 2 per cent aqueous NH_3 . The preparation consists of diluting 19.6 ml. of concentrated ammonium hydroxide (sp. gr. 0.90) to 250 ml. with distilled water, shaking a portion of this with *n*-butanol in a separating funnel and then allowing the phases to separate. The upper phase consisting of *n*-butanol saturated with the aqueous NH_3 is placed in the bottom of the chromatography jar. The lower phase consisting of aqueous NH_3 saturated with *n*-butanol is placed in a dish suspended near the top of the jar in order to maintain the proper equilibrium

conditions. This is the same solvent system as used by Way and Gailey (6).

B. *The upper organic phase from the mixture: n-butanol:ethanol:water = 27:3:10.*

C. *tert-Butyl alcohol:methanol:water = 4:5:1.*

After drying, the chromatograms were sprayed with a saturated solution of 2,4-dinitrophenylhydrazine in 2*N* HCl. This treatment soon converts the spots of the colorless aldehyde semicarbazones into the more stable colored aldehyde 2,4-dinitrophenylhydrazones. The *R_f* values obtained for several aromatic aldehydes using the three different solvent systems are shown in Table I.

TABLE I
R_F VALUES OF SEMICARBAZONES OF ALDEHYDES WITH DIFFERENT SOLVENT SYSTEMS

| Aldehyde | Solvent systems* | | |
|--------------------------------|------------------|-----|-----|
| | A | B | C |
| Cinnamaldehyde | .81 | .86 | .71 |
| Anisaldehyde | .68 | .70 | .61 |
| Piperonal | .68 | .66 | .48 |
| 3-Ethoxy-4-hydroxybenzaldehyde | .56 | .63 | — |
| Vanillin | .37 | .55 | .48 |

* See text for the compositions of the solvent systems A, B, and C.

Mixtures of these aldehydes could be separated by this method after reaction of the mixture with a reagent consisting of equimolar quantities of semicarbazide hydrochloride and sodium acetate in aqueous ethanol solution.

The method has been demonstrated with aromatic aldehydes. It also may be useful with other aldehydes and ketones. However, the 2,4-dinitrophenylhydrazones of aliphatic mono-carbonyl compounds are less highly colored and hence may be more difficult to see against the yellow background of the 2,4-dinitrophenylhydrazine reagent.

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PURIFICATION OF DDT-DEHYDROCHLORINASE FROM RESISTANT HOUSE FLIES¹

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SUMMARY

A fractionation procedure has been developed to purify the detoxication enzyme, DDT-dehydrochlorinase, from DDT resistant house flies. The method consists of acetone powdering the adult insects, purification by ammonium sulfate precipitation, dialysis, adsorption with activated charcoal and salt concentration of the resultant protein solution. Electrophoretic and ultracentrifugational studies on the final, highly active preparations present evidence of only four major protein components.

Preliminary data on a novel purification technique are presented in which DDT-dehydrochlorinase, a glutathione activated enzyme, is protected by thiourea and nonspecific protein eliminated through a series of freeze-thaw manipulations.

INTRODUCTION

Since the first reports that strains of the house fly (*Musca domestica* L.), resistant to DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane], have the ability to convert this compound to a nontoxic product, DDE [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene] (8, 11), numerous supplementary investigations have confirmed the implication of metabolism in the insecticide resistance phenomenon. The rate of dehydrochlorination has been proposed as a direct measurement of resistance in the fly (7). It has been demonstrated that the detoxication is accomplished enzymatically (13), and further studies have shown that in comparative assays the presence of the enzyme, DDT-dehydrochlorinase, is correlated with the level of tolerance in fly populations exhibiting DDT resistance (12). Conversely, adult susceptible flies do not contain detectable quantities of this enzyme. Further substantiation that a metabolic role is associated with acquired tolerance to DDT in the house fly has been supplied by research on DDT synergists (3, 14). The mechanism of action of these nontoxic compounds has been shown to be one of inhibition of the detoxifying enzyme (5, 9) which results in restoration of the effectiveness of DDT to the resistant strains. As enzyme catalyzed dehydrochlorination is an unusual biochemical reaction, a more thorough knowledge of the mechanism will be required before insect resistance to DDT can be fully understood. The present study was undertaken to purify the enzyme, DDT-dehydrochlorinase, from the resistant house fly.

¹ This work was sponsored by the Rockefeller Foundation at the University of Illinois, Urbana, Illinois, 1953-1954, under the direction of Dr. C. W. Kearns.

² Now Entomologist, Carbide and Carbon Chemicals Co. Fellowship, Boyce Thompson Institute for Plant Research, Inc.

EXPERIMENTAL

Enzyme assay. The source of enzyme used in this investigation was adult, DDT resistant house flies of the "S" strain (1). Enzyme reactions were performed in single sidearm Warburg respirometer vessels at 37° C. Four mg. of substrate, DDT, were first crystallized from a stock acetone solution onto 500 mg. of glass beads, 15 to 60 microns in diameter,³ in the body of the vessel. After delivery of the enzyme into the flask, the volume was adjusted to 2.5 ml. with 0.137 molar phosphate buffer, pH 7.4. Gassing of the reaction flasks for 15 minutes with nitrogen was carried out simultaneously with equilibration to the bath temperature. Introduction of 3 mg. of glutathione in 0.5 ml. of buffer from the sidearm marked initiation of the assay, and 90 minutes was the routine reaction time.

All assays were made in duplicate. The described purification procedure represents a culmination of the most efficient manipulations derived from trials of various fractionation techniques on over 50 individual preparations of enzyme from the acetone powder stage. The final method was tested a minimum of five times.

One unit of enzyme is defined as the quantity of enzyme required for the catalytical dehydrochlorination of 1 μ g. of DDT under conditions of the assay. The specific activity of DDT-dehydrochlorinase is expressed as units of enzyme per mg. of protein.

Analytical. Quantitative analyses of the DDT-DDE mixtures were made by first treating the reaction mixture with 3 ml. of conc. H₂SO₄. This terminated the reaction, and prevented later emulsification. After the solution cooled, 8 ml. of cyclohexane were added. The vessel was then stoppered and agitated vigorously for two hours on a wrist-action shaker to complete extraction. One-ml. aliquots of the cyclohexane layer were diluted to 21 ml. with the solvent and absorbance measured at 241 and 260 m μ with a Beckman DU spectrophotometer. Standard calculation methods for a two component system were then applied to yield (a) the level of DDT, which reflects efficiency of the extraction, and (b) the amount of the substrate enzymatically converted to DDE. The assay and analysis procedures have been described in detail (12).

Determination of the protein content of enzyme solutions was made by two methods. Prior to the first ammonium sulfate precipitation, the protein was estimated by micro-Kjeldahl nitrogen; corrected for nonprotein nitrogen on tungstate-precipitated, protein-free filtrates (2, p. 820). Later measurements were made spectrophotometrically by measuring the light absorption at wave-lengths 260 and 280, and correcting for the nucleic acid content (16).

Electrophoretic studies were made on a 1 per cent protein solution at

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0.5° C., in phosphate buffers, pH 6.5 and 7.5, ionic strength 0.1 (4). Sedimentation patterns were obtained on the same preparations. The ultracentrifugation and electrophoresis determinations were kindly performed by members of the Biochemistry Dept., University of Illinois.

PURIFICATION PROCEDURE

Acetone powders. Although fractionation of fresh breis can be carried out, for this work it was advantageous to accumulate active enzyme in the form of acetone powders prepared as the different generations of flies reached the adult stage. A number of flies under carbon dioxide narcosis or immobilized in the cold room were weighed and added to 10 volumes of acetone (weight:volume), precooled to -10° C. Homogenization in a refrigerated Waring Blendor for one minute was followed by filtration on a cold Büchner funnel. After washing the precipitate with several volumes of cold acetone, the wet filter cake was removed before any air penetrated the cake. Drying of the powder was accomplished in a vacuum desiccator where the solvent was removed under reduced pressure. Activity of DDT-dehydrochlorinase can be maintained in such preparations for at least 18 months if samples are stored in air-tight bottles under refrigeration.

Ammonium sulfate precipitation. To solubilize the enzyme, aliquots of the acetone powders were stirred into cold, deionized water at a 1:15 ratio (weight:volume). The resulting slurries were held at 1° C. to keep proteolytic action and darkening caused by the enzyme, polyphenoloxidase, to a minimum. All of the succeeding manipulations were made at 1° C. unless otherwise specified. After steeping for 2 to 3 hours, the mixtures were squeezed through a double thickness of cheesecloth to remove the chitinous fragments; the remaining insoluble components were removed by centrifugation.

The volume of the supernatant was measured and solid ammonium sulfate added to 30 per cent of saturation. Adjustment of the pH to 4.0 was then made by dropwise addition of 0.5*N* HCl. The resulting precipitate, red from the eliminated eye pigment, was sedimented by centrifugation and discarded.

Dialysis. The supernatant liquid from the previous stage was next placed in a Visking membrane and dialyzed against deionized water. Further denaturation of nonspecific protein was evidenced by a heavy flocculent precipitate which formed in the tubing as the salt was removed. No change in activity of the DDT-dehydrochlorinase was effected by the separation of this precipitate. Substitution of a 0.02 molar sodium acetate buffer for the water dialysate suppressed precipitation.

Carbon treatment. When the protein solution was free of ammonium sulfate, it was transferred to a beaker and permitted to equilibrate with room temperature. After determination of the protein concentration, 5

weight equivalents of Norite (neutral-A), powdered charcoal were added, the mixture thoroughly stirred and filtered through a double thickness of Whatman #1 paper.

Concentration. Up to this point, purification was achieved by holding the volume approximately constant and removing nonspecific protein. Concentration of the DDT-dehydrochlorinase activity proved to be the least efficient step in the fractionation. The most satisfactory results were obtained with 75 per cent ammonium sulfate saturation which yielded about one-half of the total activity in the precipitate. Only 6 per cent of the potential activity could be accounted for in the discarded supernatant. Variations in the salt content or pH, addition of the ammonium sulfate in solution as opposed to the solid, or alcohol precipitation, all failed to produce a more effective separation. A possible explanation of this phenomenon may be advanced by the recent finding that DDT-dehydrochlorinase is subject to a "concentration effect."⁴ Concentrated solutions of pure enzyme appear to elicit less activity per mg. of protein than preparations in a more dilute state.

Denaturation with thiourea. A large number of investigations have been carried out concerning the influence of urea, guanidine hydrochloride and related substances on proteins, and they have furnished valuable information regarding the processes of protein dissociation and protein denaturation. These compounds have been shown to be effective denaturing agents, functioning by partially unfolding certain protein molecules, and in many instances resulting in coagulation of the denatured proteins (6). Curiously, these findings have received little or no recognition in protein fractionation and isolation procedures.

During the later studies on purification of DDT-dehydrochlorinase, it was deemed expedient to introduce an inhibitor which would block the action of polyphenoloxidase. This troublesome enzyme is quite active in insect breis and darkens all preparations in which it is present. It has also been reported to attack aromatic moieties of other protein components in solution (10). A 1 per cent concentration of thiourea was therefore incorporated into the acetone used in preparation of the powder. It was noted subsequently that when solutions prepared from these powders were frozen and later thawed, they characteristically contained heavy precipitates of denatured protein. No loss in activity of DDT-dehydrochlorinase was discernible when this precipitate was removed by centrifugation, and assays were comparable to those made prior to freezing.

A stepwise comparison of the protein content and specific activity obtained in fractionating slurries of the regular acetone powder and powder prepared with thiourea exhibited no distinct advantages in purification of

⁴ Personal communication from Dr. Herbert Lipke, Dept. of Entomology, University of Illinois, Urbana, Ill., 1956.

either preparation. However, limited tests, in which protein solutions were repeatedly frozen with acetone and dry ice and then thawed in a water bath at 37° C., indicated that a simplified purification scheme may be possible. In one fractionation experiment, the first protein solution with thiourea was repeatedly frozen, thawed, and centrifuged eight times. The final manipulation yielded an enzyme solution with a specific activity of 215. In a second trial, this technique was performed 12 times. Little loss in the detoxifying enzyme was experienced and the specific activity was increased to 238. Although the concentration of these solutions was not determined, and physical studies were not performed, the final level of specific activity in the thiourea-freeze-thaw preparations suggested that the resultant degree of purity may be roughly equivalent to that obtained by the more laborious route previously described. A marked decrease in the activity of DDT-dehydrochlorinase invariably occurred when this procedure was attempted with powders lacking thiourea.

RESULTS

A balance sheet of the results of a typical fractionation is presented in Table I. These calculations represent purification of a 75 g. (dry weight) sample of acetone powder. Lack of homogeneity in the final solution is apparent, but physical studies indicate that only four major components are present. Four charge species were demonstrable by electrophoresis at both pH 6.5 and 7.5 (Fig. 1) and, on a weight criterion, an equal number of peaks were evidenced by ultracentrifugation (Fig. 2).

Samples of the purified solution, dehydrated by lyophilization, maintained a constant level of activity for a minimum period of a month when stored under anhydrous, refrigerated conditions.

No apparent role could be assigned to free or loosely bound trace metals as a result of these studies. Preparations, in all stages of purification, exhaustively dialyzed against 1 per cent ethylenediamine tetra-

TABLE I
PURIFICATION OF DDT-DEHYDROCHLORINASE

| Procedure | Total volume, ml. | Total protein, mg. | Units of enzyme* | Specific activity* | Per cent yield |
|---|-------------------|--------------------|------------------|--------------------|----------------|
| Whole fly homogenate | — | 300,000 | 375,000 | 1.2 | 100 |
| Acetone powder extract | 1,125 | 39,375 | 275,625 | 7 | 73 |
| 30% (NH ₄) ₂ SO ₄ precipitation | 965 | 2,316 | 212,300 | 91 | 57 |
| Dialysis | 1,335 | 2,136 | 213,600 | 100 | 57 |
| Charcoal treatment | 1,280 | 665 | 160,000 | 240 | 43 |
| 75% (NH ₄) ₂ SO ₄ concentration | 20 | 390 | 78,500 | 201 | 21 |

* See "Enzyme assay" section for definition of terms.

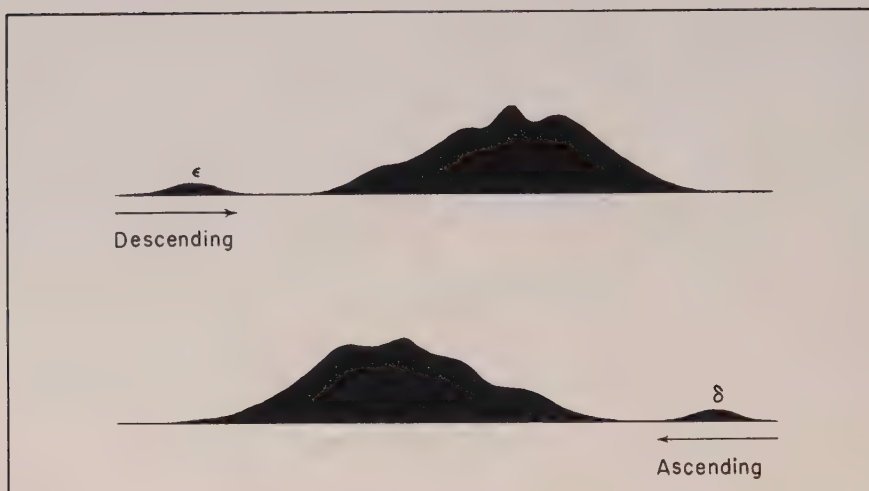


FIGURE 1. Electrophoretic pattern of purified solution of DDT-dehydrochlorinase. Protein concentration, 1%. Phosphate buffer, pH 7.5; ionic strength, 0.1. Current, 20 milliamps. Duration of run, 150 minutes. Temperature, 0.5° C. The stationary delta and epsilon boundaries are marked accordingly.

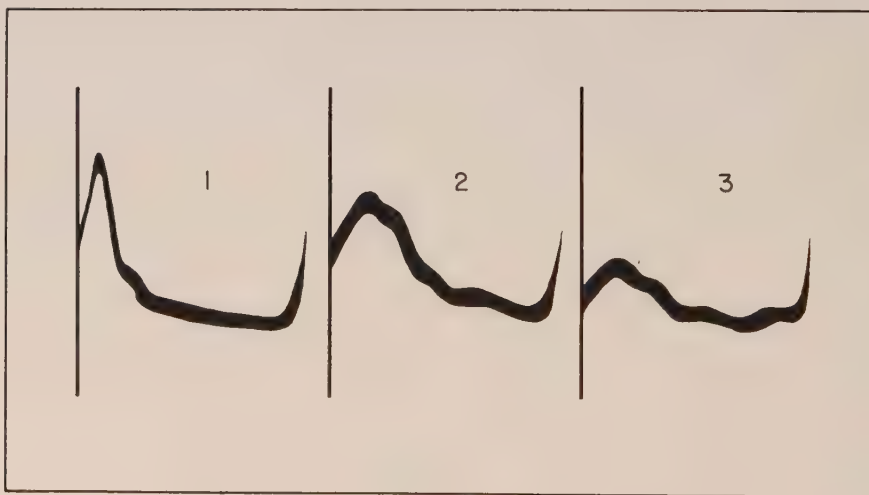


FIGURE 2. Ultracentrifugation pattern of purified solution of DDT-dehydrochlorinase. Protein concentration, 1%. Phosphate buffer, pH 7.5; ionic strength, 0.1. Traced exposures 1, 2 and 3 were taken at 1, 16 and 32 minutes respectively after full speed was attained. Speed, 59,400 r.p.m. (258,000 \times g.). Temperature of rotor, 24.9° C.

acetic acid showed no diminution of activity. Cofactors, other than glutathione, were also apparently nonessential to *in vitro* activity of DDT-dehydrochlorinase. Additions of adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN), concentrated dialysates, boiled aqueous extracts of whole fly or enzyme powder, all failed to augment the enzyme activity.

Attempts to determine the isoelectric point of DDT-dehydrochlorinase in a mixed protein solution by using the drug, Suramin sodium (Naphuride sodium; Bayer 205)⁵ (15), were unsuccessful. This enzyme falls into the class which is strongly inhibited by Suramin at pH 7.4. A 10^{-3} molar concentration of Suramin in a preparation containing 405 enzyme units elicited a 60 per cent inhibition.

A more pure enzyme solution will be required before complete characterization of the DDT-dehydrochlorinating enzyme can be accomplished and further elucidation of the detoxication mechanism can be achieved.

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⁵ Generously supplied by Sterling-Winthrop Research Institute, Rensselaer, N. Y.

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GROWTH RESPONSE OF PHYSIOLOGIC DWARFS OF *MALUS ARNOLDIANA* SARG. TO GIBBERELLIC ACID¹

LELA V. BARTON

SUMMARY

Lanolin preparations or aqueous solutions of gibberellic acid have been found to promote the growth of physiologic dwarfs produced from nonafter-ripened embryos of *Malus Arnoldiana*. Such growth is characterized by the extension of internodes, resulting in the elimination of the dwarfed condition. The number of leaves and nodes were not affected by the chemical under the conditions of the present tests. This is the first chemical shown to induce extended growth of physiologic dwarfs.

INTRODUCTION

Gibberellic acid is a metabolic product of the fungus, *Gibberella fujikuroi* (Saw.) Wr. [conidial stage *Fusarium moniliforme* (Sheld.)]. Some of the history of the discovery of this product is given by Brian *et al.* (2). The fungus is soil borne and is the cause of a disease of rice seedlings, not uncommon in Japan. A characteristic early symptom of the disease is the elongation of the shoot, so that, in an infected crop, diseased plants are much taller than healthy ones. In later stages of the disease, fungal invasion causes necrosis of the basal tissues and the seedlings become weak or die. According to Brian *et al.* (2), Kurosawa showed in 1926 that symptoms of overgrowth could be induced in rice seedlings by applying cell-free filtrates from liquid cultures of *Gibberella fujikuroi* to the roots. This was confirmed by other Japanese workers. An active material, Gibberellin A, was isolated in pure form by Yabuta and Hayashi in 1939 (2).

Gibberellic acid was isolated by Curtis and Cross (5) in 1954 from the culture filtrates of *Gibberella fujikuroi*. Cross (4) showed gibberellic acid to be a tetracyclic dihydroxy-lactonic acid. Borrow *et al.* (1) have published some observations on the production and isolation of gibberellic acid.

Gibberellic acid has been demonstrated to possess plant growth promoting properties. Brian *et al.* (2) reported that it caused extended growth of wheat, peas, oats, various grasses, clover, cucumber, tomato, linseed and dwarf bean (*Phaseolus*). Growth was affected when the material was applied to the plants through the roots, locally to leaves or stems in lanolin, sprayed on foliage, or even if applied to the seed as a dry dressing before sowing. A more recent abstract (13) describes rapid elongation of stems of a wide variety of horticultural, agronomic and forest tree species

¹ This experiment was sponsored by Eli Lilly and Company, Indianapolis, Ind.

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following application of gibberellic acid as a 1 per cent lanolin paste. Only slight increases were obtained in two species of pine and white spruce.

Brian and Hemming (3) and Lockhart (12) found that the growth of dwarf peas was significantly increased by the application of gibberellic acid to the plants. The effect of gibberellic acid and gibberellins on the growth of genetic dwarfs was determined by Phinney for varieties of corn (14, 15). He showed that normal growth could be induced in dwarf mutants of maize by the addition of gibberellic acid. However, some mutants were not affected by the acid. In six corn mutants, where single genes regulated dwarfing, four responded to gibberellic acid by normal growth, one did not respond, and the other showed only slight response. Phinney concluded that the reason for dwarfism can vary depending on the particular gene controlling the expression of the dwarfing character.

Kato (10) has recently shown that gibberellin has growth effects different from those of auxin. It reduces the growth inhibition caused by concentrated solutions of auxin and by some other growth inhibitors such as maleic hydrazide. He states, however, that the essential differences in physiological effects of gibberellin and auxin are not yet known. He made his studies on elongation, water uptake, and respiration of pea-stem sections.

In view of the elongating effect on plants of gibberellic acid, a test was conducted to determine whether this material would cause the growth of physiologic dwarfs from nonafter-ripened seeds of *Malus Arnoldiana* Sarg. Intact seeds of this species require pretreatment in a moist medium at 5° C. for at least four weeks for complete after-ripening. Intact seeds planted in soil without pretreatment fail to produce seedlings while after-ripened seeds give 90 per cent seedling production. However, some of the embryos excised from untreated seeds expand and begin to grow on moist filter paper in the laboratory. When transplanted to soil, these embryos fail to grow normally, producing dwarfish plants. Excised embryos from several other species of Rosaceae show a similar response (6, 7). The dwarf condition can be avoided by exposing the seeds or seedlings to low temperature or certain light conditions (8). Auxin treatment has been without effect. Also, no other chemical has been found to permit normal growth of these dwarfs.

The present paper is a preliminary report on the first phase of a study of the effect of gibberellic acid on dormancy in plants.

MATERIALS AND METHODS

A sample of gibberellic acid was provided by Eli Lilly and Company. This was used to treat seedlings produced from excised embryos of non-after-ripened seeds of the crab apple, *Malus Arnoldiana* Sarg. The seeds were collected in October, 1954, cleaned of pulp and stored dry in a cold room until they were used for this experiment.

Seeds were soaked in tap water for 24 hours or longer. The embryos were then excised and placed on moist filter paper in diffuse light in the laboratory. There the cotyledons turned green and some of the embryos germinated. After the root was about 10 mm. long, each seedling was planted in soil in a 3-inch pot which was placed in the greenhouse. Some of the seedlings served as untreated controls while others received gibberellic acid, either in lanolin or in water solution.

A lanolin preparation was made by placing 5 mg. of gibberellic acid in 0.5 g. of lanolin. The lanolin was melted, then the crystalline gibberellic acid was added and stirred thoroughly. While the lanolin was still soft, a small drop of the mixture was applied to the base of each cotyledon of each growing embryo at the time it was transferred from filter paper to soil. Also, 0.1 ml. of water containing 10 μ g. gibberellic acid was applied to the base of other cotyledons at the time of planting. Single applications were made in all cases.

RESULTS AND DISCUSSION

The growth of seedlings with and without gibberellic acid treatment planted in soil the same day was measured 14 days later. From 2 to 5 leaves per plant with corresponding internodes had been produced at that time regardless of treatment. However, the lengths of the stems above the ground and below the cotyledons as well as the lengths of the individual internodes were greater for those treated with gibberellic acid than for the controls. The total height of the plants, measured from the soil to the tip of the terminal expanding leaf, was also greater after treatment with gibberellic acid. The average height of the plants treated with gibberellic acid was 28.4 mm. (range 10 to 65 mm.) as compared to an average of 16.4 mm. for the untreated plants (range 10 to 24 mm.). This same general effect was to be noted when 20-day-old untreated seedlings were compared with 22-day-old seedlings treated with gibberellic acid in lanolin. In this case, the average heights of 8 untreated and 8 treated plants were 23.8 mm. (range 15 to 38 mm.) and 44.1 mm. (range 23 to 68 mm.) respectively.

The variable effect of the gibberellic acid may have been due to the lack of homogeneity of the lanolin mixture.

The type of growth of the seedlings is shown in Figure 1. Figure 1 A pictures 14-day-old seedlings. The untreated one on the left was about average height (16 mm.) while the treated one on the right was the tallest in the series (65 mm.). In Figure 1 B, 20- and 22-day-old seedlings of somewhat less than average height are shown (untreated, 20 mm.; treated 38 mm.). It will be noted that the extension of the internodes and total height of the plants was accompanied by the production of smaller, lighter green leaves.

Application of the lanolin preparation of gibberellic acid to the cotyle-

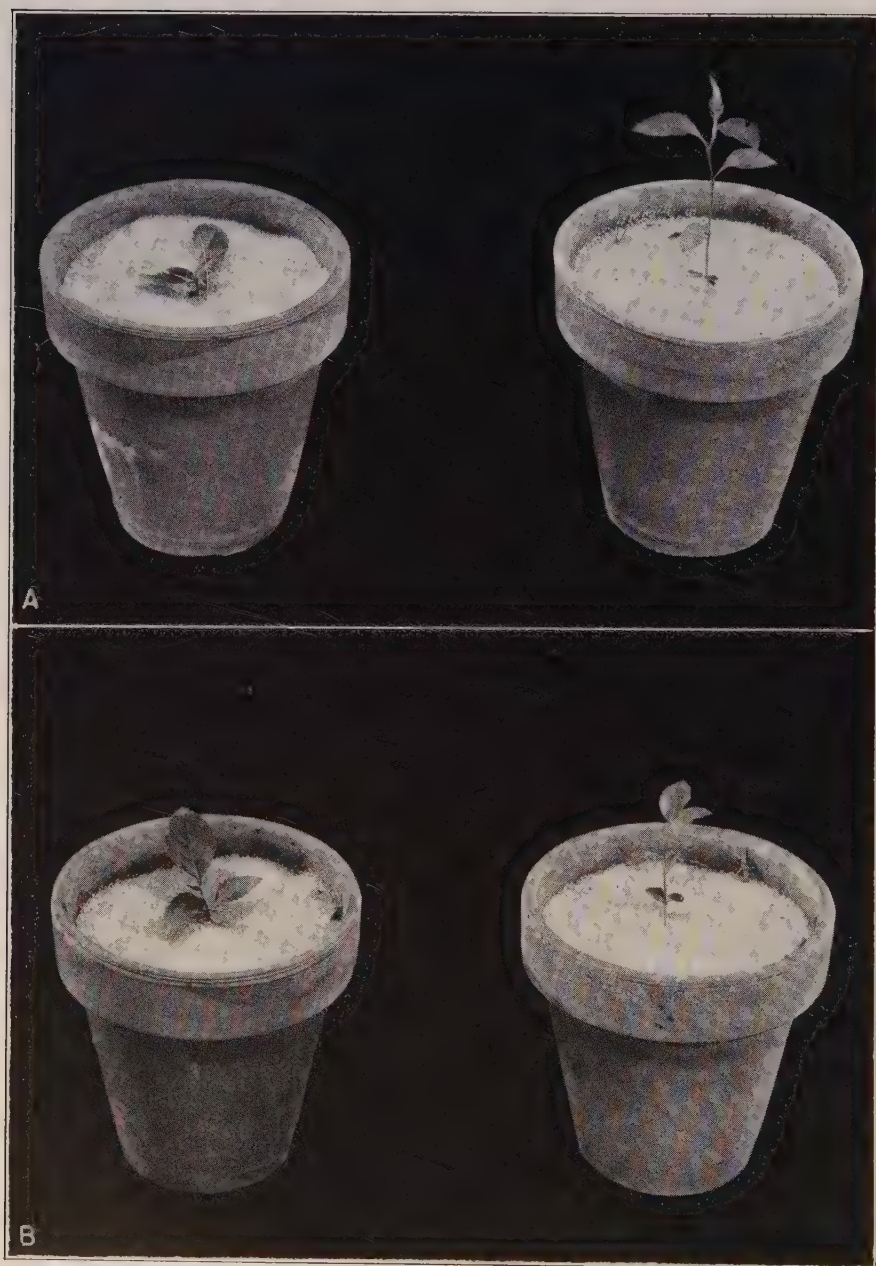


FIGURE 1. (A) 14-day-old seedlings of *Malus Arnoldiana*. Left: untreated. Right: treated with lanolin preparation of gibberellic acid. (B) Left: 20-day-old untreated seedling. Right: 22-day-old seedling treated with lanolin preparation of gibberellic acid.

dons of young seedlings seemed to have an immediate effect upon elongation of the internodes (Fig. 1). However, 0.1 ml. of water containing 10 μ g. of gibberellic acid applied to the base of the cotyledons at the time the germinating seedlings were planted in the soil was more effective for later growth. This is shown in Figure 2 which pictures such a seedling 43 days after treatment and planting in the soil. A 53-day-old dwarf seedling from



FIGURE 2. Left: 53-day-old *Malus Arnoldiana* seedling germinated on filter paper moistened with 0.2 p.p.m. 3-indoleacetic acid. Right: 43-day-old seedling treated with 10 μ g. gibberellic acid in 0.1 ml. water.

an embryo germinated on filter paper moistened with 0.2 p.p.m. 3-indoleacetic acid is shown for comparison.

With increasing age up to about 50 days, internodes of untreated seedlings began to elongate so that the heights of the plants approximated that of those treated with gibberellic acid. This was to be expected since continued elongation of pea seedlings has been shown to depend on a renewal of the gibberellic acid supply when the original application is small (3). Also, there is a tendency of the untreated dwarfs to elongate during the long days of summer (7).

The fact that low temperature and extra light are the two external conditions which have been found to cause growth of physiologic dwarfs

(8), and that gibberellic acid also has this effect, points to a relationship among these three factors. Other workers have shown similar interactions. Lang (11) found that application of a mixture of Gibberellin A with gibberellic acid caused stem elongation and flowering in noncold-treated plants of the biennial, *Hyoscyamus niger*. The chemicals did not entirely replace the long-day requirement for flowering, however. A mixture of Gibberellin A and gibberellic acid has been found to substitute for red light to stimulate germination of lettuce seeds (9). The similarity of the elongation of plants following gibberellic acid treatment to the light response, etiolation, makes these findings even more significant.

Gibberellic acid is different in its growth effect from auxin (10 and Fig. 2) but much might be learned from a study of combinations of the two types of material.

It is obvious that the gibberellic acid alters the growth habit of *Malus Arnoldiana* seedlings causing the elongation of the dwarfs produced by embryos which have not had pretreatment at low temperature, i.e., non-after-ripened embryos. In view of previous failures to bring about such elongation by the use of chemicals, this may represent a forward stride in the elucidation of the dormancy mechanism. It should be kept in mind that these "physiologic" dwarfs may be distinct from the genetic dwarfs which were the subject of Phinney's researches (14, 15). On the other hand, it is possible that the low temperature pretreatment requirement for germination and normal growth also results from biochemical control by genes.

Further work along this line is in progress and will include the effect of gibberellic acid on physiologic dwarfs of other species such as apple and peach, as well as on the after-ripening of intact seeds of these species. Other approaches to the problem are also contemplated. A special study will be made of photoperiodic effects in connection with the use of gibberellic acid.

Although gibberellin and gibberellic acid have been isolated from the fungus, *Gibberella fujikuroi*, only, they may be components of many other materials. For example, West and Phinney (16) have found gibberellin-like factors in young seeds of bean, corn, peas, lupine, and tobacco.

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A COLORIMETRIC METHOD FOR THE DETERMINATION OF 2,4-DICHLORO-6-(*o*-CHLOROANILINO)-*s*-TRIAZINE, AND RELATED COMPOUNDS

H. P. BURCHFIELD AND ELEANOR E. STORRS

SUMMARY

A method is described for the analysis of 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine and related compounds which depends upon reacting them with pyridine and measuring the colors developed when the solutions are made alkaline with aqueous sodium hydroxide. Quaternary pyridinium salts are first formed which apparently undergo ring opening to form intensely colored Schiff bases. The colors fade rapidly, but reproducible results can be obtained if absorbances are read two minutes after the addition of the alkali and compared to standards run under the same conditions. The absorption maximum obtained on 2,4-dichloro-6-anilino-*s*-triazine and its derivatives having a chlorine atom substituted in the *ortho*, *meta*, or *para* position of the benzene ring is at 440 m μ . Color intensity is enhanced by about 40 per cent when the pyridine reagent is saturated with glycine and repressed by about 10 per cent in the presence of *M*/30 phosphate buffer. The optimum concentration of the triazine is about 2 μ g. per ml. Interference from colored pigments extracted from green plants and fungus spores can be eliminated almost completely by carrying out the reaction in anhydrous pyridine, diluting with petroleum ether, and extracting with water. An aliquot of the aqueous extract is then made alkaline to develop the color. The quaternary salts are extracted quantitatively, while the plant pigments remain in the hydrocarbon phase. Plant extracts contain materials which react very rapidly with the triazines to yield products which do not produce intense colorations on treatment with pyridine and sodium hydroxide. The method is thus potentially useful for investigating the reaction rates of the triazines with cellular constituents and metabolic intermediates.

INTRODUCTION

Wolf, Schuldt and Baldwin (8) have shown that many anilino and phenoxy derivatives of *s*-triazine having active halogen atoms in the heterocyclic ring are capable of inhibiting the germination of fungus spores. Since many of these compounds are stable in aqueous media, and undergo relatively simple reactions with metabolic intermediates containing amino and sulfhydryl groups (2), it was believed that they would be useful for studying the effects of halogen substitution and isomerism on biological activity. Although more than 80 compounds were reported upon (7), these present investigations were confined to 2,4-dichloro-6-anilino-*s*-triazine, and its derivatives having a chlorine atom in the *ortho*, *meta*, and *para* positions of the benzene ring since these materials were shown to be highly effective in preventing spore germination and controlling certain foliage diseases of higher plants.

Before this investigation could be undertaken it was necessary to have

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available a sensitive analytical method that could be used to determine the uptake of these compounds from dilute aqueous solution by fungus spores, and to measure their reaction rates with metabolic intermediates.

It was found that *s*-triazine derivatives with active halogen atoms substituted in the heterocyclic ring react readily with aqueous or anhydrous pyridine to give water-soluble compounds that become intensely yellow in the presence of alkali. This is probably due to the formation of quaternary pyridinium salts followed by ring opening similar to the results described by Zincke (9) for the reaction of 2,4-dinitrochlorobenzene with pyridine. Similar procedures have been used for the determination of compounds such as chloroform (3), and trichloroethylene (1), although the reaction mechanisms are not necessarily the same.

This method has an unusual advantage in that compounds which are predominantly soluble in hydrocarbons are converted to water-soluble derivatives, thus permitting their separation from plant pigments by a single extraction. Furthermore, color formation takes place only with the unreacted compounds which should make it possible to estimate the amounts of these materials which combine with cellular constituents.

MATERIALS AND METHODS

Chemicals. Samples of 2,4-dichloro-6-anilino-*s*-triazine, 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine, 2,4-dichloro-6-(*m*-chloroanilino)-*s*-triazine, and 2,4-dichloro-6-(*p*-chloroanilino)-*s*-triazine prepared by the method described by Schuldt and Wolf (7) were dissolved in hot benzene and filtered to remove insoluble impurities. The solutions were then decolorized with Norite and the compounds crystallized. They were then recrystallized from benzene until the melting points were constant (Table I). Stock solutions containing 500 μ g. per ml. were prepared in acetone so that on dilution of 1 ml. with 99 ml. of water an aqueous solution (or suspension) containing 5 μ g. per ml. was obtained. Lower concentrations were obtained by serial dilution of the acetone stock solutions followed by dilution with water as described above. The pyridine-glycine reagent used for the analysis of aqueous solutions of the triazines was prepared by saturating a

TABLE I
PHYSICAL AND ANALYTICAL CONSTANTS OF 2,4-DICHLORO-6-ANILINO-*s*-TRIAZINE
AND DERIVATIVES

| Property | Derivative | | | |
|---|------------|------------------|------------------|------------------|
| | Unsub. | <i>o</i> -Chloro | <i>m</i> -Chloro | <i>p</i> -Chloro |
| Melting point ($^{\circ}$ C., uncorr.) | 137.5 | 157.5 | 131.8 | 187.5 |
| Molar absorptivity (pyridine-glycine reagent) | 76,400 | 76,600 | 78,200 | 77,700 |
| Molar absorptivity in presence of buffer | 67,300 | 69,400 | 70,800 | 70,800 |

70 per cent by volume solution of pyridine in water with glycine and filtering off the excess. The 7*N* sodium hydroxide solution was prepared by dissolving 280 grams of the analytical reagent grade chemical in water and making the volume up to 1 liter. Analytical reagent grade pyridine, petroleum ether (b.p. 30–60° C.), and 3*N* sodium hydroxide were used for the analysis of the triazines in extracts from plants and spores.

Analysis of aqueous solutions. Five ml. aliquots of aqueous solutions of the triazines, either in the presence or absence of *M*/30 phosphate buffer at pH 7.0, were pipetted into 50-ml. beakers and 1 ml. of pyridine-glycine reagent added. After 20 minutes, 1 ml. of 7*N* sodium hydroxide was added to develop the color, and the solution was transferred immediately to a 1-cm. Corex cell. Absorbance measurements were made at one-minute intervals on a Beckman model DU spectrophotometer against a reagent blank at a wavelength of 440 *mμ*, and the value at two minutes after the addition of alkali interpolated. The amount of triazine in solutions of unknown concentration was calculated with reference to a standard solution of the compound under investigation prepared in the same manner and at about the same time by using the equation

$$\mu\text{g. per ml. in unknown} = \frac{g \times A_u}{A_s} \quad (1)$$

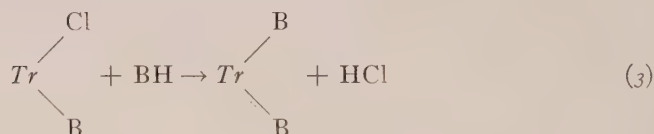
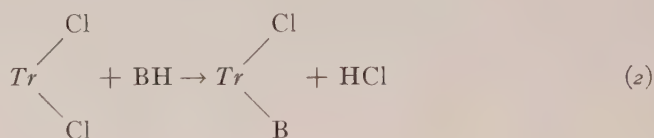
where *g* is the concentration of the standard in $\mu\text{g. per ml.}$, *A_u* is the absorbance obtained on the unknown, and *A_s* is the absorbance obtained on the standard solution. The laboratory temperature was maintained at 25° C. or lower at all times to prevent excessive fading of the color.

Analysis of plant extracts. The amount of free triazine in solvent extracts from fungus spores or green plants was determined by evaporating the extract in a 50-ml. beaker and adding 2 ml. of analytical reagent grade pyridine. After 20 minutes the pyridine was diluted with 5 ml. of petroleum ether and transferred to a 30-ml. separatory funnel. The beaker was washed with an additional 5 ml. of petroleum ether, and the solutions combined and shaken vigorously with exactly 10 ml. of distilled water measured from a pipette. The emulsion was allowed to break, and the water layer centrifuged 5 to 10 minutes in an International Clinical Centrifuge in order to clarify it. A 2-ml. aliquot of the water extract was then pipetted into a 1-cm. absorption cell and 1 ml. of 3*N* sodium hydroxide solution added. The absorbance at 440 *mμ* was determined two minutes after the addition of the alkali as described previously against a blank prepared from an equivalent amount of plant material which had not been treated with the triazine. The amount of compound in the unknown was computed from the absorbance obtained on a standard solution of the compound treated in the same manner. The average blank obtained from 300 mg. of conidia of *Neurospora sitophila* (Mont.) Shear & Dodge was

0.068 when measured against the reagent blank. Results on recoveries will be reported in forthcoming publications.

EXPERIMENTAL RESULTS

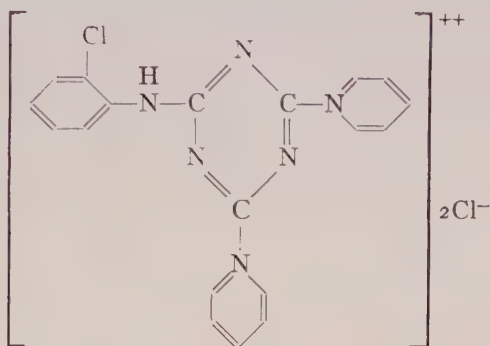
The most characteristic reaction of 2,4-dichloro-6-anilino-s-triazine and related compounds is the replacement of the halogen atoms in the triazine ring by compounds containing nucleophilic groups. The reaction is stepwise, and while replacement of the first chlorine occurs readily at room temperature, the second usually requires the application of heat (5). If the symbol *Tr* is used to designate the heterocyclic radical and BH some compound containing a nucleophilic group, the reactions can be written as



Since Schuldt and Wolf (7) had shown that the derivative with a chlorine atom substituted in the *ortho* position of the benzene ring was the most effective for the control of certain foliage diseases, most of the preliminary work was carried out with this compound. Attempts were made to develop a method of analysis by reacting it with an aromatic amine containing a group which could be converted to a chromophore. Early attempts using primary and secondary amines were unsuccessful, but it was later found that 2-aminopyridine in benzene solution would react to give a product which, when extracted with aqueous alkali, produced a brilliant yellow color in the aqueous phase. Further work showed that this was not dependent upon the primary amino group since pyridine gave a similar reaction either in benzene or water. Evidently the reaction took place at the ring nitrogen with the formation of a quaternary salt which was essentially colorless in neutral solution, but became deeply colored in alkali.

The reaction product with pyridine was prepared on a large scale using benzene as a solvent. It was deliquescent and had surface active properties, but was not stable enough to permit ultimate analysis. However, when the reaction was allowed to take place in water it was found that both chlorine atoms were eliminated since AgCl was precipitated in

stoichiometric amounts on the addition of AgNO_3 . Hence the structure of the compound in neutral solution is probably



Further evidence that the reaction takes place in two steps can be deduced from the curve relating color intensity in terms of molar absorptivity to time of reaction in aqueous 10 per cent pyridine solution (Fig. 1). The color increased rapidly to a maximum and then decreased to a constant value. Evidently an intermediate product was formed which gave a more intense color on the addition of sodium hydroxide than did the final com-

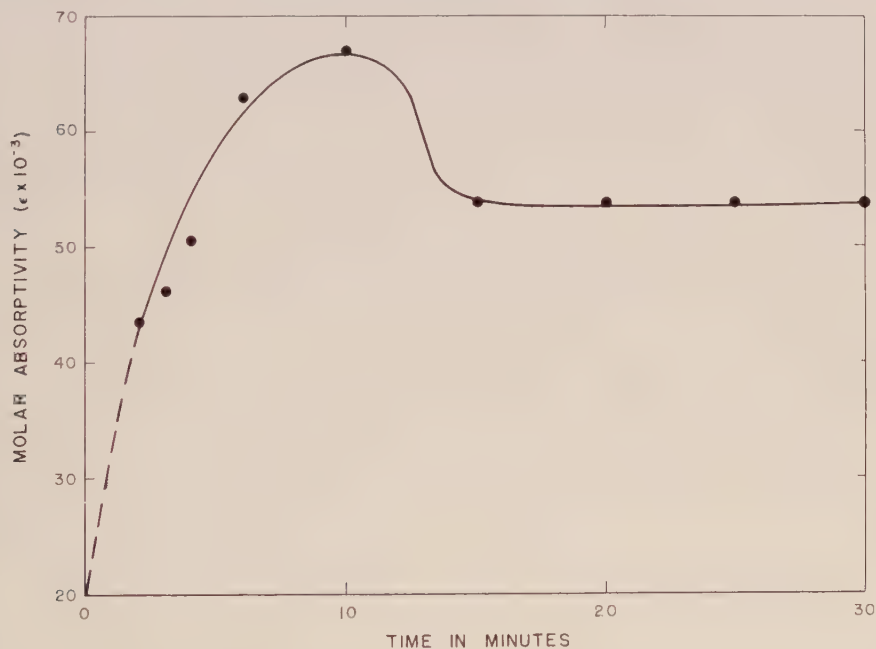


FIGURE 1. Effect of time of reaction of 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine with pyridine on intensity of color produced in 1*N* sodium hydroxide solution.

pound. It was found that the reaction product could be kept in aqueous pyridine for several hours before appreciable deterioration took place. However, once the color was developed by the addition of sodium hydroxide, it faded at a rate of 3 per cent per minute at 25° C. At higher temperatures fading took place more rapidly so it was necessary to measure color in an air-conditioned room maintained at 25° C. or less. Readings were arbitrarily made two minutes after the addition of sodium hydroxide

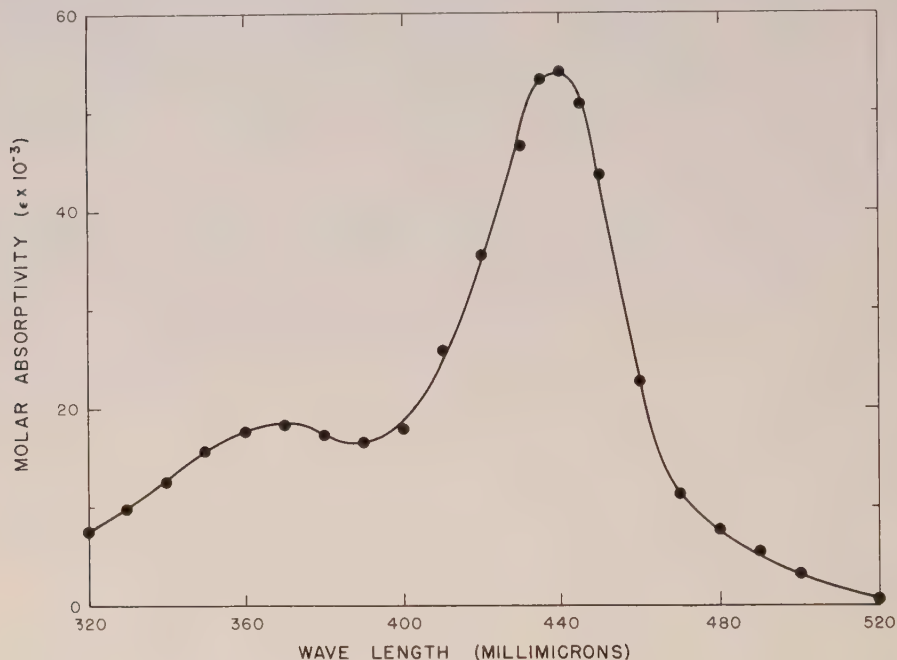


FIGURE 2. Absorption spectrum of reaction product of 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine with pyridine in the presence of 1*N* sodium hydroxide.

to allow time for adjusting the spectrophotometer. In making analytical measurements, readings were taken at one-minute intervals and the two-minute value interpolated in order to avoid chance errors in instrument settings.

Although fading of the color took place too rapidly to permit high accuracy, it was possible to obtain an absorption spectrum of reaction product with a Beckman model DU spectrophotometer by using a series of freshly prepared solutions (Fig. 2). Four to five readings at different wave length settings were taken on each solution at various times after mixing, and corrected to color intensity at two minutes by adding or subtracting 3 per cent per minute from the observed values. This assumed that fading occurred uniformly in all regions of the spectrum which is not

necessarily correct. However, the errors involved are probably small. Molar absorptivities were calculated with reference to the molecular weight of the triazine since the exact composition of the reaction product was unknown.

The concentration of alkali was found to be an important factor (Fig. 3) in determining color intensity and to a lesser extent the concentration of pyridine. Consequently all measurements were standardized at a final

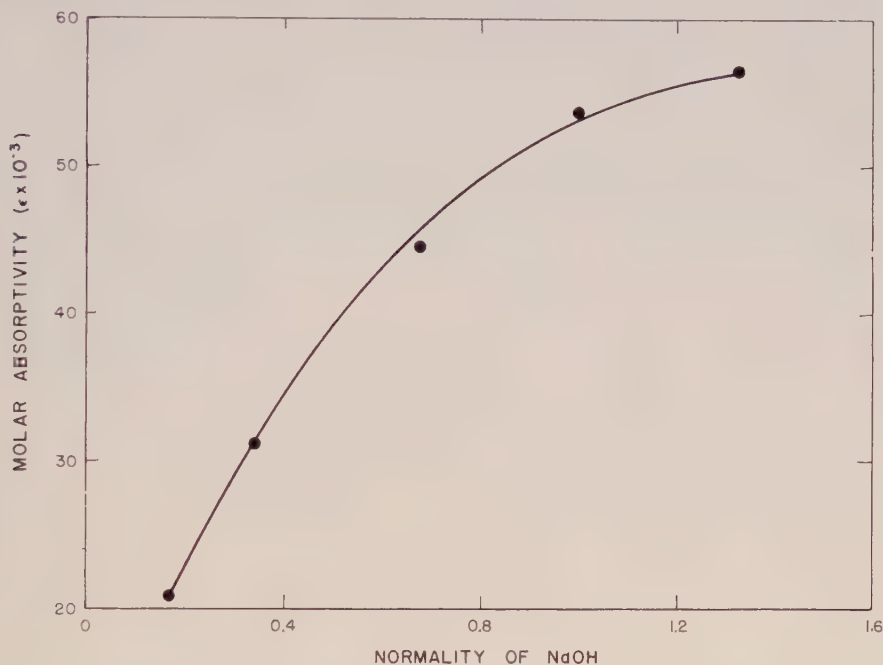


FIGURE 3. Effect of concentration of NaOH on the intensity of the color of the reaction product of 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine with pyridine.

concentration of 1*M* sodium hydroxide and 10 per cent by volume of pyridine, since small variations would not be critical in this range, and intense color combined with compatibility of the mixture could be achieved.

Several other factors were found to affect the intensity of the color. When the reaction was carried out in distilled water, the molar absorptivity at the maximum (440 m μ) was found to be 54,000 (Fig. 2). However, when attempts were made to use the method in the presence of the amino acid, glycine, the color intensity was considerably increased although the absorption maximum remained the same (Fig. 4). This was also found to be the case with cysteine and alanine. Since the method was intended for measuring rates of reaction with these compounds, the pyridine reagent was saturated with glycine in order to produce the maximum amount of

TABLE II
CHANGES IN COLOR INTENSITY OBTAINED ON ADDING 1 ML. OF WATER OR 5 PER CENT
GLYCINE AT DIFFERENT TIMES AFTER THE ADDITION OF PYRIDINE TO
2,4-DICHLORO-6-(*o*-CHLOROANILINO)-*s*-TRIAZINE

| Material added | Time after pyridine addition (minutes) | Absorbance in test No. | |
|----------------|---|------------------------|-------|
| | | 1 | 2 |
| Water | c | 0.640 | 0.652 |
| Glycine | 0 | 0.910 | 0.900 |
| Water | 2c | 0.662 | 0.654 |
| Glycine | 20 | 0.602 | 0.590 |

color possible. The maximum and color stability were found to be equivalent to those obtained in the absence of glycine except that the absorptivity was 40 per cent higher. The reasons for this enhancement in color are unknown. The glycine must in some manner affect the course of the reaction since a slight decrease in intensity was obtained when the glycine was added after the reaction with pyridine was complete (Table II).

The presence of inorganic salts was also found to affect color development, since the intensity was reduced by 10 per cent in the presence of *M*/₃₀ phosphate buffer at pH 7.0 (Table I). However, it was possible to

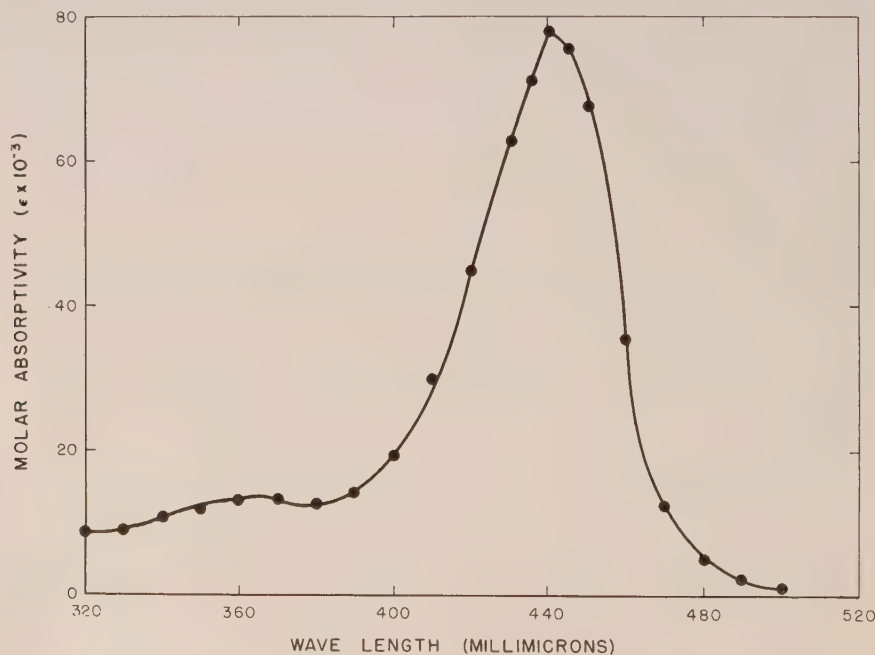


FIGURE 4. Absorption spectrum of reaction product of 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine with pyridine-glycine reagent in the presence of 1*N* sodium hydroxide.

obtain reproducible results under a wide variety of conditions by comparison with standards run under the same environment as the unknowns.

The color produced in this reaction is very intense and could be used to detect the triazines in solutions containing as little as 0.2 μ g. per ml. Results were reproducible to within ± 1.5 per cent in experiments run during the same time period and under the same conditions. The color followed Beer's law satisfactorily so it was possible to compute the amount of triazine in an unknown without recourse to a calibration curve. This was essential, since day-to-day variability was as high as 10 per cent owing to temperature effects. The method was found to be satisfactory for all of the 2,4-dichloro-6-arylamino-*s*-triazines examined, and hence was applicable to comparative studies of the uptake of these materials by fungus spores and reactions with metabolic intermediates. The absorption maximum for the unsubstituted, *o*-chloro, *m*-chloro, and *p*-chloro compounds were all at 440 $m\mu$. The intensities of the colors differed slightly (Table I), but it was possible to measure them in the same concentration range.

It was found that interferences from pigments extracted from green plants and fungus spores could be avoided by carrying out the reaction in anhydrous pyridine. The pyridine was diluted with petroleum ether and extracted with water. The pigments remained in the petroleum ether phase while the triazine reaction product was obtained in the aqueous phase. An aliquot of the water extract was then made alkaline to develop the color. The color was somewhat less intense than in the preceding method probably due to partitioning of the pyridine between the petroleum ether and water. However, Beer's law was followed satisfactorily (Fig. 5).

It was not feasible to extract the petroleum ether directly with sodium hydroxide solution since the color faded while the emulsion was breaking and some plant pigments were extracted by alkali.

In this procedure a compound which was essentially insoluble in water and soluble in hydrocarbons was converted to a compound which was highly soluble in water and insoluble in hydrocarbons. This drastic change in properties should have made it possible to obtain satisfactory analyses in the presence of most interferences likely to occur in plant materials. However, during experiments designed to test this method for the recovery of known amounts of triazine from plant and spore extracts it was found that recoveries were always low, but usually by a constant amount when tested with the same extract. Since the triazines were known to react with nucleophilic compounds, it was suspected that materials of this type might be causing the diminution in color. This was demonstrated to be the case when it was found that pretreatment of the extracts with acetyl chloride before the addition of triazine resulted in satisfactory recoveries. Presumably the acetyl chloride reacted with these compounds, and so protected the triazine.

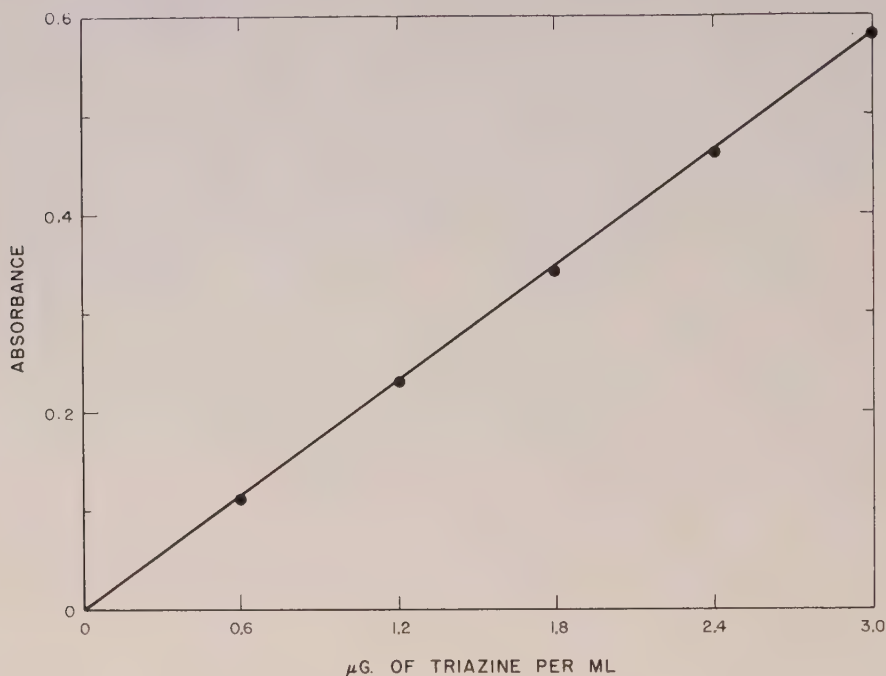


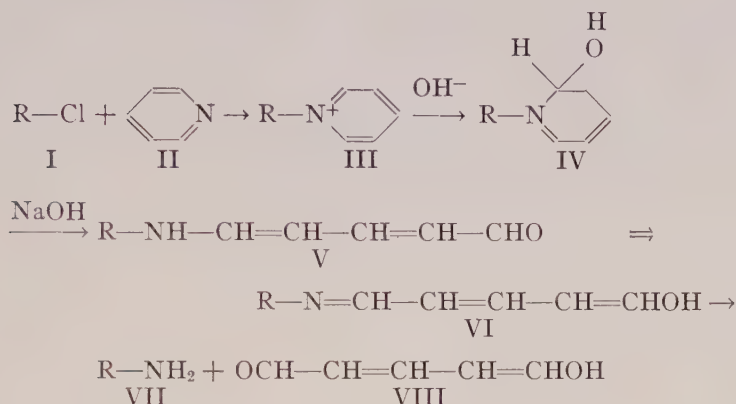
FIGURE 5. Calibration curve for the estimation of 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine by the petroleum ether extraction method.

These experiments served to show that this method might be very useful for studying the uptake and fixation of materials of this type by fungus spores since it is highly sensitive and semi-specific for the unreacted compounds. Thus it should be possible to study not only uptake, but also the capacity of the spores for combining with the chemical.

DISCUSSION

Although the compositions of the yellow compounds obtained on adding alkali to the triazine-pyridine reaction products have not been established experimentally, it seems likely that they are Schiff bases of glutamic dialdehyde (4, p. 426) formed by cleavage of the pyridine ring. This reaction was studied by Zincke (9) who investigated the structure of the red compound formed on treating 2,4-dinitrophenylpyridinium chloride with alkali. At first it was believed to be the carbinol base (IV), but it now seems more likely that it is the enol form of the open chain structure (VI), since this completely conjugated system would be expected to show strong absorption of visible light.

The rapid fading of color observed in the case of the triazines could be ascribed to hydrolysis to a melamine derivative (VII) and glutamic

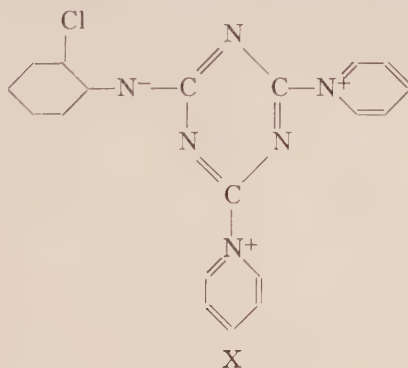


dialdehyde (VIII). At first it seemed possible that the color-deepening observed on incorporating glycine in the reaction mixture could be caused by the formation of a double Schiff base (IX) by reaction of the aldehyde group of (V) with the amino group in glycine.



However, color deepening was not observed when the glycine was added to the mixture after the reaction with pyridine was complete, but before the addition of alkali. This seems to preclude this explanation unless it is assumed that ring opening and Schiff base formation can take place in the absence of the base. This is very unlikely since the double Schiff base would be expected to be highly colored in the absence of alkali, in view of its conjugated structure, and this was not observed experimentally.

Another explanation for color formation that was considered was the possibility that the labile amino hydrogen atom on the triazine-pyridinium chloride would ionize in alkaline solution to yield an inner salt (X).



However, this interpretation is not satisfactory since 2,4-dichloro-6-(*N*-methylanilino)-*s*-triazine in which the amino hydrogen is replaced by a methyl group also gives an intense coloration in the pyridine-sodium hydroxide reaction. The most likely mechanism for color development appears to be opening of the pyridine ring, although the reasons for color enhancement on the addition of glycine to the reagent are not apparent.

This method appears to offer considerable promise for the analysis of pesticides containing active halogens, and in fact has already been successfully applied to an experimental compound of another class, although the reaction conditions required are different. Commercially available materials which have been observed to give color reactions in this test include chloranil, dichlone, lindane, heptachlor, chlordane, and toxaphene (6). Reaction conditions, color intensities, and properties differ considerably, and it is uncertain whether any of them can be used for quantitative determinations. Other compounds such as DDT, methoxychlor, aldrin, dieldrin, etc. have failed to react under any of the conditions employed.

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Note

GLASS FLOWMETER WITH INTERCHANGEABLE
CAPILLARIES

R. MAVRODINEANU

During fumigation experiments with sulfur dioxide (SO_2) and hydrocarbon gases to determine effects on plants, there was need for a sensitive, flexible, and rugged apparatus to control the dispensing of these two gases into the fumigating cabinets. Therefore, a flowmeter fulfilling these requirements was designed.¹ The flowmeter (Fig. 1) consists of a large-scale concentric differential manometer, M, connected to the capillary, C, and tube, U, through ground glass joints. The delivery can be varied over a wide range by using capillary tubes of different diameters and lengths. Capillaries 10 cm. long with an inside diameter of 0.3 and 0.4 mm. proved satisfactory in recent fumigation experiments. Tube U is secured to its ground glass joint by rubber bands placed around hooks, H.

The gas to be metered enters the side tube on the left and, after passing through tube U and capillary C, following the arrow, leaves by the side tube on the right. The resulting pressure is measured in the differential manometer, M, by the displacement of the colored mineral oil used as indicating liquid.

The flowmeter can be calibrated for various purposes. When SO_2 was used in fumigating experiments, the differential readings were related to the concentration of this gas found in the fumigating cabinet. For example, with a capillary of 0.3 mm. inside diameter, a length of 100 mm. and a differential reading of 180 mm., a concentration of 1 p.p.m. SO_2 was obtained in a cabinet having a volume of 200 cu. ft. in which the air was displaced once per minute. Similarly, a differential reading of 100 mm. gave a concentration of 0.5 p.p.m. SO_2 . Under the same conditions the results were reproducible in different tests. In these experiments a mixture of 10 per cent SO_2 and 90 per cent dry nitrogen was passed through the flowmeter.

¹ Made by the Yonkers Laboratory Supply Co., Yonkers, N. Y., according to specifications.

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(See illustration on next page)

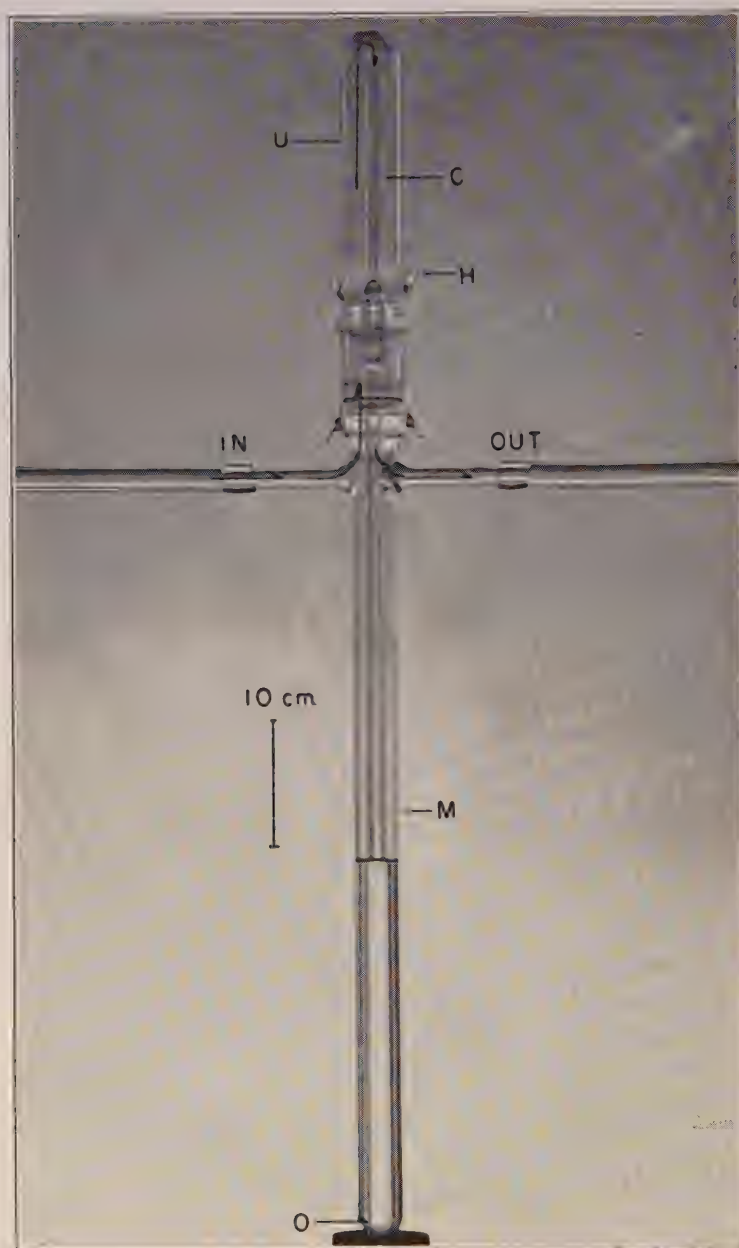


FIGURE 1. Pyrex glass flowmeter with interchangeable capillaries. C, capillary; H, one of four hooks used for tightening the connection between tubes U and M by means of rubber bands (not shown); M, concentric differential manometer; O, opening (approximately 3 mm. in diameter) at the bottom of the inner tube for liquid circulation; U, tube.

A METHOD FOR DETERMINING THE INCIDENCE OF CLUBROOT INFECTION IN NUTRIENT CULTURES

ELMER T. PALM AND GEORGE L. MCNEW

SUMMARY

In order to distinguish between the effects of soil treatments on the initiation of infection and the overgrowth response of crucifers to *Plasmodiophora brassicae*, a simple, reliable method was developed for inducing infection in sand culture and measuring its incidence. Seeds were germinated in washed silica sand infested with resting spores and zoospores and supplied with a balanced nutrient solution containing 2 milligram equivalents of calcium per liter. Seedlings were removed after six days, placed in acetocarmine, and the area 0.5 to 1.5 cm. below the seed attachment was examined microscopically for the brilliantly stained plasmodia.

Any increase or decrease in available calcium from 2 mg. eq. per liter caused a sharp decrease in infection. This suggests that one of the major effects of liming soils is to increase calcium supplies beyond the optimum for infection.

Soil treatments are used to control clubroot of cruciferous crops caused by *Plasmodiophora brassicae* Wor. Although liming of soils to pH 7.2 as recommended by Chupp (1) and many others is the most widely used, it is known (2, 3, 6) that nutrient balance influences the severity of clubbing. The soil treatments conceivably may operate either by affecting the resting spores or zoospores, changing the resistance of epidermal cells to invasion or modifying the host's ability to respond to infection and produce overgrowths.

The effect of nutrient balance on disease development cannot be properly interpreted without differentiating between the effects on persistence of inoculum, incidence of infection and gall development from each infection site. In order to analyze the effect of certain data obtained on nutrient cultures of cabbage, broccoli and turnip in the greenhouse, a method was sought for measuring incidence of infection. The technique of Samuel and Garrett (5) for staining plasmodia in root hairs and other epidermal cells seemed ideal for this purpose but gave very erratic results on plants grown in nutrient solutions and sand cultures. Studies were undertaken to develop a method for use primarily on sand cultures. Some of the essential features of the method are described in this report.

GENERAL EXPERIMENTAL METHODS

Cultures of foliage turnip (*Brassica rapa* L.) var. Shogoin and cabbage (*Brassica oleracea* L.) var. Golden Acre were maintained in nutrient solution or sand. A medium fine grade of washed white silica sand (Ottawa Silica flintshot) was held for 24 hours in 18 per cent hydrochloric acid

solution, rinsed in running tap water until all acid was removed and then rinsed in distilled water.

In most experiments the sand was moistened with a nutrient solution containing reagent grade potassium nitrate, calcium nitrate, monobasic potassium acid phosphate, magnesium sulfate and trace elements prepared according to Robbins (4). Where calcium content was to be varied a basic nutrient solution containing .002*M* sodium nitrate, .002*M* potassium sulfate, .002*M* magnesium nitrate, .00178*M* monobasic sodium phosphate, .00022*M* dibasic sodium phosphate, .00025*M* ammonium sulfate, and trace elements as recommended by Robbins was used. Calcium was added by replacing the sodium nitrate with calcium nitrate up to 2 mg. eq. per liter and calcium sulfate thereafter.

Inoculum was added to the nutrient solution at the rate of approximately five million resting spores per milliliter. Inoculum was obtained directly from clubbed roots freshly harvested or held in storage for less than a month at 5° C. The clubs were macerated in a Waring Blendor, the suspension filtered through cheesecloth to remove plant debris, and the spores were washed at least three times by centrifugation and resuspension in distilled water.

Plants were removed from the culture six days after exposure to the inoculum, washed in tap water and placed in 1 per cent aqueous aceto-carmine. They were left in the stain for 1 to 30 days until they could be examined under the 16 mm. objective of a microscope for plasmodia. Data were taken on the number of infected epidermal cells and root hairs in the zone 0.5 to 1.5 cm. below the seed attachment.

EXPERIMENTAL RESULTS

Nutrient Solution

Seedling turnips grown in moist sand were transferred to nutrient solution 7 to 10 days after the seeds were sown. The seedlings were supported on cotton plugs in a perforated flat cork stopper. There were only 2 infection loci on 20 seedlings.

This method was considered unsatisfactory because few of the infected roots produced clubs. Usually the clubs were decayed by bacterial contaminants and many of the young roots sloughed off. Attempts were made to eliminate the bacteria associated with *Plasmodiophora brassicae* by surface sterilizing clean clubs in 10 per cent Clorox (0.525 per cent sodium hypochlorite) for 15 minutes, rinsing them in sterile distilled water and rinsing the spores repeatedly by centrifugation and resuspension. However, bacteria always were present in sufficient numbers to interfere with plant development and club growth so attention was focused on the use of sand culture.

Sand Culture

The staining technique of Samuel and Garrett (5) effectively revealed infection on plants grown in soils but repeatedly failed in exploratory tests made on plants grown in sand supplied with either Robbins' solution or water. It was assumed that some organic material in soil was necessary for germination of resting spores or survival of zoospores until they could penetrate the root hairs. As shown by the data on the first experiment in Table I, mixtures of soil and sand gave about four times as much infection as sand alone and were approximately as satisfactory as undiluted soil.

The idea of an organic supplement in soil, however, was disproved by use of a subsoil clay. As shown by the second experiment, a mixture of clay

TABLE I

INCIDENCE OF INFECTION ON SHOGIN TURNIP SEEDLINGS GROWN IN SAND, SOIL AND CLAY MEDIA AND WATERED WITH ROBBINS' NUTRIENT SOLUTIONS AT pH 5.9

| Composition of medium (%) | | | No. of plasmodia/cm. of radicle | |
|---------------------------|------|------|---------------------------------|-------------|
| Sand | Soil | Clay | First test | Second test |
| 100 | 0 | 0 | 6 | 4 |
| 0 | 100 | 0 | 25 | 32 |
| 0 | 0 | 100 | — | 4 |
| 90 | 0 | 10 | — | 18 |
| 50 | 0 | 50 | — | 17 |
| 90 | 10 | 0 | 22 | — |
| 10 | 90 | 0 | 28 | — |

and sand was much more effective than sand even though somewhat less satisfactory than soil. The light infection of plants in pure clay is attributed to the poor growth of seedlings.

While investigating the role of soil colloids, studies were also made on the availability of calcium. This factor was found to be more important than the proportion of colloids in sand. Satisfactory infection was obtained on plants in sand as soon as the calcium level was dropped from the 10 mg. eq. per liter in Robbins' solution to about 2 mg. eq. per liter. Typical data on the occurrence of infection on roots exposed to the basic nutrient solution adjusted to 5 levels of calcium are given in Table II.

In sand where calcium was readily available and not absorbed on colloidal material, maximum infection was obtained from 2 mg. eq. of calcium per liter. Infection falls off sharply below this level and somewhat more gradually as the level is increased to 4 or 8 mg. eq. per liter. These data have been confirmed repeatedly by other tests with turnip, cabbage and broccoli, the only deviation being that the level of calcium for optimum infection may be modified appreciably by varying the ratio of other nutrients.

Effect of Seedling Size on Infection

Considerable variation in infection on plants growing in sand cultures was observed. This was correlated in several cases with size and vigor of growth during the first six days after germination. Since growth during this period is influenced by quantity of food available from seed, experiments were made on the effect of seed size on incidence of root infection.

TABLE II
EFFECTS OF AVAILABLE CALCIUM ON INITIAL INFECTION
OF TURNIP ROOTS IN SAND CULTURE

| Calcium concentration mg. eq. per liter | Number of infections per cm. of root |
|--|---|
| 0 | 2.4 |
| 1 | 8.2 |
| 2 | 42.8 |
| 4 | 13.1 |
| 8 | 16.8 |
| Min. sign. difference at 5% level | 24.7 |
| Min. sign. difference at 1% level | 31.1 |

A sample of turnip seed was passed through a series of screens so as to separate them into classes less than 1.5 mm. in diameter, 1.5 to 2.0 mm., and more than 2 mm. All three lots germinated satisfactorily but seedlings grew directly in proportion to the size of seeds. The plants from small seed had 56 infections per centimeter of root as compared to 125 infections on the intermediate group and 180 on the more vigorous seedlings from large seeds.

The severity of infection also may be suppressed by growing the plants under conditions that are not favorable for their development. In a test replicated 4 times the cultures were held at 20°, 25°, and 30° C. for 6 days. The mean number of loci infected were 12.0, 389.2 and 247.2 respectively. However, seedlings held for 13 days at 15° C., to permit further growth, had 205.5 plasmodia per cm. of root. Seedlings held at 10° C. were practically free of infection (0.16 plasmodia/cm.) after 18 days.

These data indicate that optimum conditions for infection prevail at about 25° C. The plasmodia in plants at 25° and 30° C., for some unexplained reason, stained a much more brilliant red than those at lower temperatures.

DISCUSSION

A simple method for determining the effect of soil conditions on the incidence of root infection of crucifers by *Plasmodiophora brassicae* has been developed. By use of sand cultures it is possible to regulate the nutrient supply and the balance between elements during the initial stages of infection. The concentration of calcium in the nutrient solution appears to

be of more importance than soil colloids or organic matter in determining the severity of infection.

Maximum infection of turnips occurred when nutrient solutions contained about 2 mg. eq. per liter of calcium. The number of parasitic establishments at pH 5.9 declined rapidly when supplies of calcium were either reduced or increased from this level. These data suggest that liming a soil may reduce the severity of clubroot as much by supplying calcium ions as by creating an alkaline or neutral condition to impede the fungus.

Most cabbage soils probably have moderate supplies of calcium and it is conceivable that the addition of one to three tons of lime might raise the concentration of calcium above the optimum for infection. However, only detailed studies on the interrelationship of calcium and other nutrient elements will provide an answer to the problem of nutritional effects on clubroot. Now that a suitable technique is available such studies can be made.

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RELATION OF DIFFERENT GASES TO THE SOAKING INJURY OF SEEDS. III. SOME CHEMICAL ASPECTS

LELA V. BARTON AND JEAN MACNAB

SUMMARY

Bean seeds were soaked in water supplied with oxygen, carbon dioxide, nitrogen or air for various periods. Their capacity to germinate was partly or entirely lost depending on the gas supplied and length of treatment. Oxygen was most injurious to the seeds and carbon dioxide protected them from injury. Of twenty-four different chemical solutions tested for preventing oxygen injury, only hydrogen peroxide and, to a lesser extent, crude catalase were effective. Hydrogen peroxide also prevented injury by other chemicals during the soaking periods. Injured seeds absorbed excessive amounts of water.

Results of chromatographic analyses showed that injury by oxygen was accompanied by an increase in glutamic acid, and a reduction in arginine and γ -aminobutyric acid in both beans and soaking solution. Aspartic acid disappeared entirely under all conditions after 24 hours' soaking. Other amino acids noted were asparagine, serine, alanine, leucine, methionine sulfone and pipecolic acid.

The amounts of amino acids leached from the seeds while they were soaking could not account for the injury. Also the addition of amino acids to the soaking solutions did not improve germination though in cases where germination did occur some beneficial effects were noted on the subsequent growth of the seedlings.

Sucrose, raffinose, and an unknown sugar were present in all bean extracts. In general, after 3 hours of soaking, oxygen-treated seeds appeared to contain more of all sugars than carbon dioxide-treated ones. After 24 hours carbon dioxide-treated seeds contained much more of each sugar than the oxygen-treated seeds, due, at least in part, to excess leaching during extended soaking in the presence of oxygen. Sorbose, not detected in extracts of dry or soaked seeds, was found in all leachates.

Changes in IAA and *p*-aminobenzoic acid content of the beans and of the water in which they were soaked could not be related to changes in germinative capacity.

INTRODUCTION

Seeds of *Phaseolus* are injured by prolonged soaking in water at room temperature. It was shown (1) that a current of oxygen passed through the soaking water increased the injury, while carbon dioxide supplied during the soaking process prevented injury. The carbon dioxide not only protected bean seeds against injury by soaking in water, but also prevented toxicity to embryos by nutritive inorganic salt solutions and by selenium salts and 2,4-dichlorophenoxyacetic acid. Excessive absorption of water has been found to accompany injury. Polyvinylpyrrolidone in 5 or 10 per cent solutions retarded the absorption of water by seeds and reduced soaking injury in the presence of oxygen, but had no effect in the presence of carbon dioxide (2).

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The present tests were undertaken to determine whether there are other substances which will prevent the injurious effect of oxygen or differences in amino acid, sugar, and growth regulant contents of both seeds and soaking water. The bush bean, *Phaseolus vulgaris* L., French Horticultural Variety #5106 from W. Atlee Burpee Co., was used throughout.

PREVENTION OF OXYGEN INJURY BY THE USE OF CHEMICALS

The general method was to soak counted and weighed lots of seeds in measured amounts of solution at laboratory temperature and treat with different gases during the soaking period. Nonaerated solutions were used as controls. The gases tested were air, oxygen, nitrogen, and carbon dioxide. Air from the laboratory was pulled through the soaking solutions by means of suction. Oxygen, nitrogen, and carbon dioxide were supplied from commercial cylinders of these gases. All gases were allowed to bubble slowly through the solutions containing the seeds for the test period, then the solutions were drained from the seeds which were used for germination and growth tests and for chemical analyses.

HYDROGEN PEROXIDE EFFECTS

Among a large number of chemical solutions, hydrogen peroxide proved most effective for preventing soaking injury in the presence of oxygen. Hydrogen peroxide in concentrations of 0.5 or 1.0 per cent permitted full germination of seeds which had been soaked for as long as 16 hours in the presence of a stream of oxygen. This was in contrast to a reduction in germination to 0 to 24 per cent when the bean seeds were soaked in distilled water under similar conditions. Some of these effects are shown in Figure 1. Here, it will be seen that soaking in water supplied with oxygen for 6 hours resulted in decreased vigor of the bean seedlings produced from the soaked seeds as compared with seedlings from seeds soaked in hydrogen peroxide with oxygen or in water with carbon dioxide. This difference became more marked following soaking for 12 hours, and soaking for 24 hours in water supplied with oxygen prevented germination entirely. Soaking in hydrogen peroxide with oxygen for 24 hours reduced subsequent germination, while carbon dioxide bubbled through the soaking water permitted best germination.

Germination of seeds soaked in water or 1.0 per cent hydrogen peroxide supplied with oxygen or carbon dioxide for one of the series of tests is shown in Table I. Here, the progressive injurious effect of prolonged soaking in water supplied with oxygen is shown. On the other hand, seeds were not injured when soaked in water supplied with carbon dioxide. Also, oxygen was without any harmful effect when the soaking solution was 1 per cent hydrogen peroxide instead of water.

In the light of the water relations accompanying soaking injury (1, 2),



FIGURE 1. Effect on germination of soaking beans for 6(A), 12(B), or 24(C) hours. Twenty-five seeds each soaked. Left to right: in water with oxygen supplied; in 1 per cent hydrogen peroxide with oxygen supplied; in water with carbon dioxide supplied.

tests were made of the water absorbed by the seeds under the present conditions. These results are also shown in Table I. Again it has been demonstrated that an excessive water absorption accompanies the injury to seeds soaked in water in the presence of oxygen. This excessive absorption does not occur in hydrogen peroxide solution in the presence of oxygen, nor in either water or hydrogen peroxide solution in the presence of carbon dioxide. Excessive water absorption parallels decrease in germinative capacity.

Hydrogen peroxide also protected bean seeds from injury by 3-indoleacetic acid, *p*-benzoquinone, and 2,4-dichlorophenol. Doubtless it would protect from other substances as has been demonstrated for carbon dioxide (2).

TABLE I

EFFECT OF GASES BUBBLED THROUGH WATER AND HYDROGEN PEROXIDE SOLUTION ON WATER ABSORPTION AND GERMINATION OF BEAN SEEDS

| Soaking medium | | Percentage water absorbed* after hours of soaking | | | | Percentage germination of seed soaked for hrs. | | | |
|----------------|----------------------------------|--|-----|-----|-----|---|-----|-----|-----|
| Gas | Solution | 2 | 4 | 8 | 16 | 2 | 4 | 8 | 16 |
| Oxygen | Water | 71 | 101 | 108 | 111 | 92 | 72 | 36 | 24 |
| | H ₂ O ₂ ** | 25 | 32 | 50 | 77 | 100 | 96 | 100 | 100 |
| Carbon dioxide | Water | 17 | 29 | 45 | 84 | 100 | 100 | 100 | 100 |
| | H ₂ O ₂ ** | 22 | 29 | 46 | 66 | 100 | 100 | 100 | 100 |

* Calculated on the basis of the air dry weight of the seeds.

** 1 Per cent solution.

EFFECT OF CRUDE CATALASE PREPARATION

Somewhat less effective than hydrogen peroxide in preventing soaking injury was a crude catalase preparation (catalase 10,000 Keil units/g., Armour and Co.). Bean seeds soaked in 0.1, 0.5, 1.0, or 2.0 per cent catalase preparation saturated with oxygen germinated 80 to 84 per cent as compared with 12 per cent germination of the control lot soaked in water. Weaker concentrations were less effective. Crude catalase solutions, as well as hydrogen peroxide solutions, had no effect when carbon dioxide was bubbled through during the soaking period.

Under the conditions of these experiments, crude catalase was less effective than hydrogen peroxide in preventing injury by other chemicals. It should be pointed out that the catalase used was not pure and may have contained other substances which would affect soaking injury.

EFFECT OF OTHER CHEMICALS

Other chemicals tried but found ineffective in preventing soaking injury in the presence of oxygen were as follows:

Acetone (1.0 per cent)

Ascorbic acid (10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} M)*p*-Benzoquinone (10^{-6} M)Citric acid (10^{-1} , 10^{-3} M)Cobalt chloride (10^{-3} M)Cysteine hydrochloride (10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} M)2,4-Dinitrophenol (1×10^{-5} , 3×10^{-5} , 5×10^{-5} M)Ferric chloride (10^{-3} M)

Ferric sulfate (2 and 4 per cent)

Glutathione (10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} M)Glutathione, oxidized (10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} M)

Guaiacol (0.001 per cent)

- 3-Indoleacetic acid (10 p.p.m., 20 p.p.m.)
- 3-Indoleacetic acid-potassium salt (10^{-6} M)
- Manganese chloride (10^{-4} , 10^{-6} M)
- Penicillin (32 p.p.m.)
- Phenol (3×10^{-5} M)
- Pyruvic acid (0.001 per cent)
- Sodium arsenite (1×10^{-5} , 3×10^{-5} , 5×10^{-5} M)
- Sodium azide (10 concentrations from 1×10^{-9} to 5×10^{-5} M)
- Sulfanilamide (250 p.p.m.)
- Terramycin (32 p.p.m., 50 p.p.m.)

DISCUSSION

Treatment with hydrogen peroxide may act to raise the oxidation-reduction potential in the soaked seeds. Cysteine and glutathione which lower this potential were ineffective in preventing soaking injury. Galston and Siegel (9) reported that Mn^{++} increases the rate of peroxide genesis in etiolated pea tissues leading to increased peroxidative destruction of IAA and thus to decreased growth rate. Co^{++} worked in the reverse direction, decreasing peroxigenesis thus sparing IAA and increasing growth of 3-day-old Alaska pea seedlings. Control roots exposed to pure oxygen for 5 hours were injured, but the injury was prevented by Co^{++} solution. These authors believed that decreased peroxide levels accounted for the prevention of injury. In the present tests with bean soaking, an external supply of H_2O_2 prevented oxygen injury but manganese and cobalt solutions in the concentrations used were without beneficial effect.

Sodium azide was tried in a number of concentrations because of its effect in preventing the rotting of ungerminated seeds soaked in it. This was in contrast to the usual rotting of all seeds which did not germinate within one or two days after removal from the soaking solutions. In spite of this effect, however, no benefit of sodium azide on seeds soaked in the presence of oxygen was noted, though most of the concentrations tried had no injurious effect when carbon dioxide was passed through the soaking water.

Sodium azide and arsenite are inhibitors of oxidative enzyme systems and of growth and they have also been reported to prevent water absorption by pea seedlings, as does 2,4-dichlorophenol (10). Auxin has been found to increase the water uptake by discs of potato tuber tissue, but not under anaerobic conditions (11). These were of special interest because of the obvious differences in water absorption under the influence of different gases, and the harmful effects of the excessive absorption in oxygenated water. Neither sodium azide nor 3-indoleacetic acid prevented soaking injury in bean seeds. Also sodium azide at 3×10^{-7} M and 3-indoleacetic acid at 10 and 20 p.p.m. did not increase water uptake up to 16 hours'

soaking under the influence of either oxygen or carbon dioxide. It will be recalled that hydrogen peroxide solution reduced the absorption of water by bean seeds in the presence of oxygen and had no effect in the presence of carbon dioxide. Eyster (8) reported that the rate of water absorption by bean seeds is inversely proportional to the concentration of hydrogen peroxide in nonaerated solutions.

Although water relationships have not been elucidated, it seems evident that absorption is an important factor in the influence of oxygen and carbon dioxide on soaking injury. It is not known at the present time whether prevention of oxygen injury by hydrogen peroxide or crude catalase solutions is effected inside or outside of the seed.

FREE AMINO ACID CONTENT

The explanations which have been advanced for the deleterious effects of soaking beans have been discussed previously (1). Among these are the leaching out of soluble food reserves, digestive enzymes, and growth promoting substances (8).

Paper chromatography has been used to determine the effect of soaking bean seeds for various lengths of time under different conditions on the free amino acid contents of both beans and leachate.

CHROMATOGRAPHIC PROCEDURE (4)

Dry beans to be extracted were weighed after the seed coats had been removed. Other dry seeds in lots of five each were weighed with seed coats intact prior to soaking and treatment in 30 ml. of distilled water in the laboratory. After soaking, the seed coats were removed before extraction, air dried and their weights subtracted from the weights of the entire seeds. Dry weights of five seeds varied from 1.8830 to 3.0582 g. The amount of water left for extraction after the five seeds had soaked for the prescribed period varied from 23.8 to 29.1 ml.

Treatment consisted of nonaerated soaking in distilled water, and soaking with the addition of oxygen, carbon dioxide or nitrogen for periods of 3, 6, 12 and 24 hours.

The seeds were homogenized in 80 per cent ethanol in a VirTis '45' homogenizer,¹ centrifuged, and the residue washed three times with 25 ml. of 80 per cent ethanol. The supernatants from each sample were combined and evaporated to dryness in the hood. The residue was transferred to 50-ml. centrifuge tubes with 10 ml. of distilled water and 6 ml. chloroform and again evaporated to dryness. The residue in the centrifuge tubes was dissolved with exactly 10 ml. of distilled water and 6 ml. of chloroform, the solutions were centrifuged and 9 ml. (90 per cent) of the aqueous layer

¹ The VirTis Company, Inc., Yonkers, N. Y.

were removed with a pipette and evaporated to dryness. The final extracts were made up to 2-ml. volume with distilled water.

The samples of water which contained substances leached out of the treated beans were simply evaporated to dryness and made up to 1-ml. volume.

These extracts also were used for sugar determinations.

Extracts were applied to 20.2 cm. \times 20.2 cm. Whatman No. 1 filter paper for two dimensional chromatograms. Two and one-half microliters each of the bean extracts representing from 2.1184 to 3.4405 mg. of dry seeds and leachate extracts were used in replicates of four, in addition to mixed standard amino acids in applications of 0.5, 1.0, 3.0 and 5.0 micrograms. The dried spots were treated for 5 minutes with ammonia vapor to neutralize any acids. The papers were irrigated first with liquid phenol: water (150:30) and after thorough drying, with 2,4-lutidine:ethanol: water:diethylamine (110:40:50:2).

The papers were dried, dipped in acetone:ethyl ether (1:1) to remove excess diethylamine, dried again and then dipped in 1 per cent ninhydrin in acetone. They were developed 90 minutes at 35° C. The maximum densities of the spots were determined with the aid of a Photovolt densitometer using a 550 m μ filter for blue-violet spots and a 440 m μ filter for brown spots (asparagine). Micrograms of amino acids in the extracts were determined by comparison with standard curves.

RESULTS

Some of the results are given in Table II. Conclusions given below are based on significant differences revealed by analyses of variance of absorbance readings for replicates of 4 chromatograms for each extract.

Glutamic acid. Considering glutamic acid first, it will be noted that seeds soaked in the presence of oxygen have much more of this acid present than those soaked in the presence of carbon dioxide. After 3 hours of soaking, the amount of glutamic acid in seeds supplied with nitrogen was somewhat higher than in those nonaerated or supplied with oxygen or carbon dioxide. After 6 hours the quantity of glutamic acid was the same in the nonaerated and the carbon dioxide lots, but both of these had lower quantities than either nitrogen or oxygen, and oxygen was significantly higher than all the others. This high value for glutamic acid content of oxygen-treated seeds persisted for the 12- and 24-hour soaking periods. Also, after the 12- and 24-hour periods carbon dioxide-treated seeds had smaller amounts of glutamic acid than the others. Nonaerated and nitrogen-treated seeds occupied intermediate positions. Thus the injury to bean seeds soaked in the presence of oxygen, known to be initiated after 6 to 8 hours of soaking (Fig. 1), was accompanied by high glutamic acid content. Similarly, the use of carbon dioxide which prevents soaking injury re-

TABLE II
AMOUNT OF AMINO ACIDS IN BEAN SEEDS AND WATER AFTER SOAKING FOR DIFFERENT PERIODS IN THE PRESENCE OF VARIOUS GASES

| Amino acid | Gas | $\mu\text{G./g.}^*$ seeds after hours of soaking | | | | | | | |
|-----------------------------|-----------------|--|------|------|-----|------------------|-------|-------|------|
| | | In seeds | | | | In soaking water | | | |
| | | 3 | 6 | 12 | 24 | 3 | 6 | 12 | 24 |
| γ -Aminobutyric acid | None | 195 | 356 | 382 | 339 | 57 | 72 | 37 | 42 |
| | O ₂ | 50 | 330 | 320 | 359 | 21 | 41 | 73 | 187 |
| | CO ₂ | 54 | 324 | 579 | 586 | 0 | 61 | 30 | 311 |
| | N ₂ | 167 | 388 | 526 | 446 | 0 | 179 | 175 | 142 |
| Arginine | None | 535 | 645 | 530 | 616 | 89 | 45 | 103 | 81 |
| | O ₂ | 330 | 1015 | 581 | 494 | 0 | 18** | 15** | 0 |
| | CO ₂ | 269 | 649 | 837 | 806 | 0 | 71 | 74 | 421 |
| | N ₂ | 418 | 742 | 1201 | 739 | 0 | 74 | 158 | 0 |
| Asparagine | None | 584 | 424 | 276 | 416 | 114 | 100 | 72 | 31 |
| | O ₂ | 429 | 543 | 131 | 434 | 0 | 139 | 29 | 37 |
| | CO ₂ | 538 | 470 | 322 | 421 | 0 | 20 | 12** | 92 |
| | N ₂ | 385 | 607 | 461 | 231 | 0 | 127 | 206** | 97 |
| Aspartic acid | None | 357 | 136 | 0 | 0 | 81 | 212** | 249 | 312 |
| | O ₂ | 429 | 260 | 131 | 0 | 45 | 91 | 169 | 296 |
| | CO ₂ | 467 | 632 | 274 | 0 | 31 | 109 | 131 | 302 |
| | N ₂ | 703 | 202 | 0 | 0 | 61 | 211** | 191 | 57 |
| Glutamic acid | None | 292 | 289 | 658 | 632 | 114 | 104 | 74 | 139 |
| | O ₂ | 297 | 1157 | 770 | 823 | 39 | 56 | 105 | 202 |
| | CO ₂ | 251 | 243 | 290 | 238 | 31 | 51 | 48 | 137 |
| | N ₂ | 469 | 388 | 428 | 800 | 38 | 91 | 125 | 42 |
| Serine | None | 0 | 68 | 0 | 77 | 22 | 34 | 13 | 31 |
| | O ₂ | 0 | 142 | 145 | 120 | 8** | 15** | 16** | 26** |
| | CO ₂ | 0 | 32 | 97 | 73 | 9** | 16** | 8** | 27** |
| | N ₂ | 0 | 67 | 132 | 77 | 10 | 36 | 37 | 0 |
| Valine | None | 0 | 85 | 127 | 139 | 22** | 47 | 23** | 39 |
| | O ₂ | 0 | 212 | 116 | 165 | 10** | 24 | 20 | 45 |
| | CO ₂ | 0 | 81 | 97 | 109 | 0 | 26 | 16** | 69 |
| | N ₂ | 0 | 67 | 115 | 108 | 0 | 72 | 49 | 2** |

* Average of 4 replicates.

** Extrapolated values.

sulted in smaller amounts of glutamic acid in bean seeds. Dry seeds contained approximately the same amount (312 $\mu\text{g./g.}$) as the carbon dioxide-treated seeds at all stages of soaking.

γ -Aminobutyric acid. Nonaerated and nitrogen-treated seeds soaked three hours contained larger quantities of this acid than those treated with either oxygen or carbon dioxide (Table II). At the end of 6 hours of soaking these differences tended to disappear. After 12 hours' soaking, γ -aminobutyric acid content of both carbon dioxide- and nitrogen-treated seeds was higher than that of seeds from either of the other two treatments, and this high position was maintained by seeds soaked for 24 hours in the

presence of carbon dioxide. Dry seeds contained no measurable amount of this acid.

Arginine. Dry seeds contained 141 $\mu\text{g./g.}$ of this acid. It will be seen from Table II that arginine was formed quickly by bean seeds in the soaking process and that its appearance was somewhat more rapid under the influence of no aeration or of nitrogen than in the presence of either oxygen or carbon dioxide. After 12 and 24 hours there was an increased amount of arginine in carbon dioxide- and nitrogen-treated seeds as compared to that in oxygen-treated or with no aeration.

Asparagine. Dry seeds contained 100 $\mu\text{g./g.}$ Results of assay of seeds after soaking were variable so that no particular significance can be attached to the supply of gases during soaking.

Aspartic acid. The most striking effect to be noted in this case was the disappearance of the acid under all soaking conditions when the period was as long as 24 hours. This disappearance followed an initial rise from 140 $\mu\text{g./g.}$ which were present in the dry seeds. After 3 hours most aspartic acid was found as a result of nitrogen treatment, and after 6 and 12 hours most was found after carbon dioxide treatment.

Serine and valine. Dry seeds contained no serine and 31 $\mu\text{g./g.}$ of valine. No serine or valine was found in seeds soaked for 3 hours. With increased soaking time, however, the amount increased in all cases, but perhaps more under the influence of oxygen.

Other amino acids. Leucine was detected in dry seeds in quantities of 54 $\mu\text{g./g.}$ of dry seeds. Also, in addition to the acids discussed above, methionine sulfone was detected in considerable quantity (179 to 369 $\mu\text{g./g.}$) on chromatograms of extracts of seeds soaked for 3 and 24 hours. Apparently, alanine was present in all extracts but quantitative determinations were not made since alanine and homoserine were not separated in the solvent system used. Pipecolic acid was always present in large amounts in both bean and soaking water extracts but quantities were not measured for lack of a standard. Rf values for pipecolic acid obtained in the present tests corresponded to those reported by Zacharius *et al.* (16) who isolated and identified the acid as a constituent of dry seeds of beans. Boulanger *et al.* (5) found that pipecolic acid and arginine made up more than two-thirds of the total free amino acids of bean seeds. They also found small amounts of glutamic acid, alanine, and some unknown spots. In the present tests, at least two unknowns appeared as spots between alanine and asparagine on the chromatograms.

Radhakrishnan and Vaidyanathan (13) have reported arginine, valine, α -alanine, leucines, phenylalanine, serine, proline, tyrosine and traces of histidine, β -alanine, and α -aminobutyric acid in seeds of *Phaseolus radiatus*.

Amino acids leached out. In a comparison of oxygen and carbon dioxide effects, much more arginine and more γ -aminobutyric acid leached out

under the influence of carbon dioxide, while the reverse seemed to be true for glutamic acid. These effects were more apparent for the longer soaking periods. It will be recalled that this same relationship held for the quantities of these three amino acids present in the seeds after soaking (see above). In general, the results, especially when no aeration and nitrogen effects are taken into consideration, negate the contention that leaching of essential amino acids causes soaking injury as claimed by Eyster (7). In addition to amino acids shown in Table II, threonine, leucine, and lysine, which were not present in the soaked seeds, were in the water in which the seeds had soaked. Also large amounts of pipercolic acid and unknowns were leached out during the soaking process.

Effect of soaking solution on amino acid content. Since hydrogen peroxide has a tendency to counteract the harmful effect of oxygen, the amino acid content of seeds soaked for 24 hours in 0.5 per cent hydrogen peroxide supplied with either oxygen or carbon dioxide was determined. Controls treated with the two gases in distilled water contained the same relative amounts of amino acids as described for another experiment above, except that oxygen-treated beans contained more arginine and asparagine than carbon dioxide-treated beans.

The beans soaked in hydrogen peroxide supplied with oxygen contained larger quantities of the following amino acids than did those treated with oxygen in water: aspartic acid, serine, glutamine, threonine, and methionine sulfone. Beans supplied with carbon dioxide while soaking in water generally contained smaller amounts of all amino acids calculated except for γ -aminobutyric acid which was higher than in water plus oxygen or either of the hydrogen peroxide treatments.

Since there is so little agreement between the uninjured beans treated with carbon dioxide in water and those protected from oxygen injury by hydrogen peroxide, it appears that the amino acid content of the beans may not be directly correlated with the loss of germinative capacity.

Beans were supplied with oxygen and carbon dioxide for 24 hours in solutions of terramycin (50 p.p.m.) and sulfanilamide (250 p.p.m.) to reduce bacterial contamination. No significant differences in amino acid content as compared with those soaked in distilled water were found.

DISCUSSION

An increased amount of glutamic acid in seeds soaked in nonaerated water or in water supplied with oxygen or nitrogen during soaking as compared with those soaked in the presence of carbon dioxide, indicates a possible relation to soaking injury. The opposite, i.e., reduction in content, seems to apply to arginine and γ -aminobutyric acid where reduced amounts present in the seeds accompany injury.

Experiments were conducted to see whether supplies of these amino acids in the external solution would affect soaking injury in the presence

of oxygen and carbon dioxide. Glutamic acid solutions of 0.25, 0.5, and 1.0 per cent injured seeds soaked 24 hours in the presence of carbon dioxide, as compared with no injury in distilled water. In 0.25 per cent this injury was evident only in the rotting of ungerminated seeds, but in both of the higher concentrations germination was reduced, the seedlings were short, and the radicle tips were blunt and brown. No germination occurred after soaking in any of these solutions in the presence of oxygen.

Similarly seeds were soaked in 0.1, 0.05, and 0.025 per cent γ -aminobutyric acid and 0.5, 0.25, and 0.125 per cent arginine for 24 hours. No benefit to germination was secured when oxygen was supplied to seeds soaked in these solutions, but in the carbon dioxide series all seedlings from beans treated with the amino acids were larger 2 days after germination than those soaked in water. There was apparently a beneficial effect on seedling growth though no differences in germination were noted (all between 96 and 100 per cent).

Thus there is no clear relation between external supply of these acids and injury by soaking, though there may be some indication of this by the injury of glutamic acid to carbon dioxide-treated seeds.

The disappearance of rather large amounts of aspartic acid from seeds soaked under all conditions as the soaking period is increased, resulting in its total disappearance after 24 hours of soaking, may be of significance in seed metabolism.

SUGAR CONTENT

CHROMATOGRAPHIC PROCEDURE (4)

Aliquots of the extracts prepared for free amino acid determinations were spotted one inch from two opposite edges of $18\frac{1}{4} \times 22\frac{1}{2}$ inch Whatman No. 1 filter paper so that both edges of the paper rested in the solvent when the paper was suspended over a glass rod in the chromatography tank. The solvent was the upper phase from a mixture of *n*-butanol:acetic acid:water (40:10:50). The solvent was allowed to run overnight and the papers were dried until the odor of the solvent had disappeared.

Aniline acid phthalate failed to show the presence of glucose. Papers were sprayed with a 1:9 mixture of 1 per cent ethanolic resorcinol in 2*N* HCl and developed 10 minutes at 80° C. Pink spots corresponding with sucrose and raffinose appeared plus a strong unknown at R_f .025, and a weak unknown at R_f .14. Amounts of sucrose were calculated by determining the maximum density of the spots using the Photovolt densitometer with a 495 $m\mu$ filter. Quantitative measurements of other sugars could not be made because of the background color which developed.

RESULTS AND DISCUSSION

Dry beans contained 22.1 mg./g. of sucrose. After soaking for 3 hours in the presence of oxygen, the sucrose content had increased to 48.1 mg./g.

and in carbon dioxide there had been a slight decrease to 10.2 mg./g. After 24 hours of soaking, the sucrose content of the oxygen-treated seeds had fallen to 25.0 mg./g., while that of the carbon dioxide-treated seeds had increased to 50.3 mg./g. Thus, the amount of sucrose in the seeds treated with oxygen was approximately the same after 3 hours as that in carbon dioxide-treated seeds after 24 hours of soaking.

Extracts of the corresponding solutions in which these seeds had soaked showed ten times more sucrose leached from oxygen-treated seeds than from carbon dioxide-treated ones after 3 hours: 1.2 and 0.11 mg./g. respectively. After 24 hours of soaking the amounts leached out had increased to 5.0 and 0.33 mg./g. These results indicate that sucrose is formed quickly in bean seeds soaked under the influence of oxygen, but, as the seeds lose their viability with extended soaking, much more of the sugar appears in the leachate, while very little of the increased sucrose content leaches from carbon dioxide-treated seeds.

Raffinose was also measured in the same extracts used for sucrose determinations. The 9.2 mg./g. of this sugar found in dry seeds decreased to approximately 1.0 mg./g. with soaking under all conditions. No raffinose was found in leachate from carbon dioxide-treated seeds but 0.08 and 0.59 mg./g. leached from oxygen-treated seeds after 3 and 24 hours' soaking.

Sorbose, which was not detected in dry or soaked seeds, was found in all leachates, and in much greater quantity after oxygen than after carbon dioxide treatment.

An unidentified spot at R_f .40, probably stachyose, was present in all bean extracts and in soaking water extracts from oxygen treatment. However, none of this sugar was found in leachates from carbon dioxide-treated seeds.

In general, after 3 hours of soaking, oxygen-treated seeds appeared to contain more of all sugars than carbon dioxide-treated ones. After 24 hours, on the other hand, carbon dioxide-treated seeds contained much greater amounts of all sugars than the oxygen-treated seeds, due, at least in part, to excess leaching with extended soaking in the presence of oxygen.

INDOLE COMPOUNDS AND OTHER COMPOUNDS IN BEAN SEEDS

We have seen that an external source of 3-indoleacetic acid (IAA) was ineffective in preventing soaking injury. Experiments were initiated to determine (a) whether an excess of this or other similar materials is leached from seeds under the influence of oxygen, (b) whether the IAA in bean seeds is increased in amount upon soaking and initiation of germination, (c) whether IAA is destroyed by the presence of oxygen in the soaking water or preserved or increased in beans soaked in water in the presence of carbon dioxide, and (d) whether either oxygen or carbon dioxide hastens inactivation of IAA. Paper chromatographic procedures were employed to elucidate this problem.

CHROMATOGRAPHIC PROCEDURE (4)

Twelve hundred dry bean seeds each were placed in 2-liter vacuum flasks with 1500 ml. of distilled water. Controls were soaked without aeration. Other samples were treated by bubbling oxygen and carbon dioxide through the water. Two of the oxygen-treated samples were soaked in 1 per cent hydrogen peroxide or 1 per cent crude catalase solution instead of distilled water. These two substances had been found to protect the seeds from injury by oxygen.

Each of these treatments was allowed to proceed for 0.75, 1.5, 3, 6, 12, or 24 hours before the seeds were drained and blotted free of excess liquid. Four replicates of 50 seeds each were rolled in moist paper towels and put in moist chambers at 20° to 30° C. daily alternating temperature for germination tests. The remaining 1000 seeds were frozen rapidly in a methanol-dry ice bath and stored in tightly covered bottles in a food freezer at -17.8° C. until extracts were made. Each lot of 1000 beans to be used for extraction had an approximate dry weight of 551 grams based on an average of 30 samples which ranged from 535.5 grams to 558 grams.

The frozen seeds were ground in small amounts in a Waring Blendor with absolute ethanol using 1 liter of ethanol in all. They were allowed to stand in a cold room at -4° C. overnight. The solid material was centrifuged out and washed with 400 ml. absolute ethanol. The ethanol extracts were evaporated nearly to dryness under a stream of air.

They were then filtered through glass wool, adjusted to pH 3.0 with HCl and extracted three times with ethyl ether (200, 100, and 100 ml.). The ether was partitioned five times with 5 ml. of glucose-saturated water buffered at pH 9.0 with sodium bicarbonate and sodium hydroxide. The combined water fractions of each sample were acidified to pH 3.0 and extracted three times with 100 ml. ether. The ether extracts were evaporated to dryness and the residues made up to 1- or 2-ml. volumes with 100 per cent isopropyl alcohol. The number of microliters applied to one dimensional chromatograms were adjusted so that they represented comparable weights of dry seeds, either 10, 20, and 40 microliters or 20, 40, and 80 microliters.

The solutions in which the seeds were soaked were frozen for storage and thawed before extraction. The procedure used for the seeds to reduce interfering fatty substances was altered in this case as there was relatively little fat present in the soaking water. The volumes of water were reduced to about 100 ml. under a stream of air, their pH raised to 8.5 with sodium bicarbonate and sodium hydroxide, and they were extracted twice with 200 ml. ethyl ether. The ether was discarded. The water fractions were acidified to pH 3.0 and partitioned three times with 200 ml. ether. The ether was evaporated to dryness and the residues made up to 2 ml. with 100 per cent isopropyl alcohol.

Sheets of Whatman No. 1 filter paper ($18\frac{1}{4} \times 22\frac{1}{2}$ inches) were spotted

1 inch from the edge along two opposite edges, both of which rested in the solvent when papers were hung over glass rods in the irrigation tank. The solvent, isopropyl alcohol:58 per cent ammonium hydroxide:water (80:5:15), was allowed to run overnight. Papers were dried in a hood and developed by spraying with 1 per cent *p*-dimethylaminobenzaldehyde in 1*N* HCl at room temperature. 3-Indoleacetic acid appeared after an hour at Rf .52 as blue-violet spots and another substance, later identified as *p*-aminobenzoic acid, appeared immediately at Rf .29 as bright yellow spots. Large amounts of extraneous matter in the extracts tended to depress all Rf values.

RESULTS AND DISCUSSION

3-Indoleacetic acid (IAA). The first measurements were made with seeds which had soaked for 16 hours in water supplied with different gases. Although standards of 0.3125, 0.625, 1.25, 2.5, and 5.0 μg . of IAA made it possible to prepare a curve for quantitative estimation of the substance

TABLE III
ABSORBANCE READINGS (PHOTOVOLT DENSITOMETER) FOR CHROMATOGRAMS OF
3-INDOLEACETIC ACID (IAA) AND *p*-AMINOBENZOIC ACID (PABA) IN BEAN SEEDS
AND WATER AFTER SOAKING FOR 16 HOURS IN THE PRESENCE OF
VARIOUS GASES. APPLICATIONS OF 80 μL . REPRESENTING 17.3 G.
OF DRY BEAN TISSUE

| Chemical | Extract | Nonaerated | Air | O ₂ | CO ₂ | N ₂ |
|----------------|-----------------------|------------|-----|----------------|-----------------|----------------|
| IAA | Bean Soaking water | .34 | .48 | .21 | .18 | .23 |
| | | .04 | .07 | .07 | .07 | .10 |
| PABA | Bean Soaking water | .31 | .39 | .27 | .25 | .27 |
| | | .12 | .31 | .03 | .03 | .15 |
| % Germination* | | 97 | 96 | 4 | 97 | 95** |

* 4×100 Seeds.

** Weak seedlings.

in bean seeds, the streaking of the extracts at detectable concentrations on the chromatogram made densitometer readings unreliable. However, absorbance readings shown in Table III gave some indication of the amounts of material present. As a rough estimate of the amount of IAA present, an absorbance reading of 0.25 represents approximately 8 μg . of IAA per 100 grams of bean seeds. Although IAA was present in extracts of both the beans themselves and the water in which they had been soaked, there was no indication from these tests that its presence was related to soaking injury. Certainly the extracts of leachates were not different enough to indicate more leaching of IAA from the seeds damaged the most by soaking, i.e., under the influence of oxygen. Such differences, if they exist, should have been evident in this series, for only 4 per cent of the

seeds from oxygen treatment germinated while other treatments failed to injure the seeds.

Further tests of extracts of both seeds and soaking solution after 0.75, 1.5, 3, 6, 12, and 24 hours in water, hydrogen peroxide or crude catalase solutions supplied with oxygen or carbon dioxide showed that the IAA in the seeds increased in amount with increased period of soaking, but the relative increase remained the same for all treatments regardless of effect on germination capacity, except for the 6-hour soaking period when there appeared to be more IAA in the carbon dioxide-treated seeds. Similarly there seemed to be no relation between the amount of IAA leached out of the seed and soaking damage. Only traces were found in the water in which seeds had soaked for 6 hours or longer. The largest amounts were found in the soaking water after 3 hours. It may be that the extraction method failed to remove all of the free IAA from the seeds since there was only 25 per cent recovery when 2 mg. of IAA were added to 552 grams of beans during the grinding process.

It would not appear from the present results that there was more inactivation of IAA under the influence of oxygen than under carbon dioxide though it has been reported (15) that enzymatic inactivation takes place only in the presence of oxygen.

Also 2,4-dichlorophenol, which is known to increase the activity of indoleacetic acid oxidase (10), did not alter either oxygen or carbon dioxide influence during the soaking process.

No direct relation of 3-indoleacetic acid, whether applied in external solution, leached from the seeds, or remaining in the seeds, was found under the conditions of the present experiments.

p-Aminobenzoic acid (PABA). In the first chromatograms made for the detection of indole compounds in seeds soaked for 16 hours a deep yellow spot at R_f .29 appeared as soon as the reagent touched the paper. This was later identified as *p*-aminobenzoic acid by comparison with standards of the material and by using solvents recommended by Kelemen *et al.* (12). A picture of these first extracts is shown in Figure 2 where both IAA and PABA standards appear. The intensity of the PABA spots in the extracts is evident in spite of the streaking. Also, it will be noted that dry seeds are not shown to contain any PABA, or at least not enough to be detected by this method. The fainter IAA spots are also to be noted in this figure.

Absorbance measurements made for the PABA in both soaked seeds and soaking solutions are shown in Table III. From PABA standards, it is estimated that an optical density of .25 would represent about 4 μ g. of material per 100 grams of bean seeds. Again, as in the case of IAA, there was no clear relationship between the amount of PABA, either remaining in the seeds or leached out, and oxygen injury during soaking.

It did not seem likely that the presence of PABA was due to bacteria on the seeds or in the solutions in spite of the fact that bacteria are known to synthesize this substance. Seeds injured by oxygen treatment during soaking rot rapidly and presumably would have more bacteria, but they did not have more PABA. The same logic would apply to the soaking water from the oxygen treatment. However, experiments were conducted to see the effect of sterilization of the seeds upon PABA content. Several sterilization methods involving the use of 95 per cent alcohol and/or calcium hypochlorite were tried, but these injured the bean seeds so that



FIGURE 2. Paper chromatogram of bean seeds extracted after soaking in water for 16 hours. Spots applied from left to right were 5 μ g. *p*-aminobenzoic acid (PABA); 5 μ g. of 3-indoleacetic acid (IAA); 20 μ l. each of extracts of dry seeds and seeds soaked nonaerated or in the presence of air, oxygen, carbon dioxide or nitrogen; a repetition of above series with 40 μ l. of each extract.

germination was prevented. Beaudreau and Remmert (3), in a study of the Krebs cycle activity of particles from bean seedlings, added terramycin at 5 and 50 p.p.m. to the incubation mixtures to inhibit growth of bacteria and to establish that the activity studied was not caused by bacteria. Terramycin at 50 p.p.m. as well as sulfanilamide at 250 p.p.m. were finally chosen as the solutions to use for sterile soaking of bean seeds. Neither of these solutions harmed seeds under the influence of carbon dioxide, nor prevented injury under oxygen treatment. PABA was measured quantitatively in extracts of bean seeds soaked without aeration in distilled water, 50 p.p.m. terramycin, or 250 p.p.m. sulfanilamide. The results showed 3.565, 3.186, and 5.80 μ g./100 g. of seeds respectively, thus giving some

evidence that the formation of PABA is not dependent on bacterial growth. Seeds which had been soaked in the sulfanilamide solution had an extra yellow spot at Rf .66, which was shown later to be sulfanilamide itself.

PABA content in both seeds and soaking water was followed, for periods of soaking in water, hydrogen peroxide, and crude catalase solutions supplied with both oxygen and carbon dioxide. As in the case of IAA, the amounts increased with increased time of soaking until there was about an 8-fold increase by 24 hours, but no differences due to oxygen or carbon dioxide were noted.

PABA is known to nullify the antibiotic effect of sulfanilamide (14, pp. 66-79). Seeds were soaked in 0.25 per cent concentration of PABA, sulfanilamide and a mixture of the two for 8 hours with oxygen and carbon dioxide supplied. There was no germination after soaking in the presence of oxygen. With carbon dioxide supplied, soaking in water, PABA, sulfanilamide, and a combination of PABA and sulfanilamide permitted subsequent germination of 64, 52, 92, and 44 per cent respectively.

The significance of the presence of PABA in bean seeds is not clear since its role in the metabolism of higher plants is not known. It has been suggested that it may exist in combination with a protein, functioning as a prosthetic group of some enzyme (14, p. 13).

Biological assays have been used to demonstrate the presence of PABA in seeds of alfalfa, corn, oats, peanuts, rice, and wheat (14, p. 30) but, so far as the authors are aware, it has not been previously reported for beans. Indications from the data at hand are that bean seeds contain larger quantities of PABA than reported for other seeds.

Other indole compounds. In the seed extracts and in soaking water extracts there was a violet unknown on the paper chromatogram at Rf .14, almost totally masked by pigment from the extracts. A pink-lavender spot at Rf .33 which faded to definite dark blue was particularly evident in seeds soaked in hydrogen peroxide. A yellow spot at Rf .19 or lower (down to .096) may be allantoin known to be present in the bean plant in considerable quantity (6), but its presence was obscured by the brown color of extract.

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EFFECT OF ETHYLENEDIAMINETETRAACETIC ACID ON NITROGEN METABOLISM AND ENZYME PATTERNS IN SOYBEAN PLANTS¹

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SUMMARY

Experiments were carried out to determine the effect of increasing concentrations of disodium ethylenediaminetetraacetate (Na_2EDTA) on nitrogen metabolism and enzyme patterns of sunflower and soybean plants grown in solution cultures. Toxicity symptoms of ethylenediaminetetraacetic acid (EDTA) on sunflower plants were manifested by slight mottling of leaves. On soybeans, these symptoms were accompanied by severe twisting of young leaflets and interveinal bronzing of older leaflets. With soybeans maximum height and fresh weight yields were obtained at 10 p.p.m. of Na_2EDTA , while maximum dry weight yield was obtained at 5 p.p.m. Concentrations of Na_2EDTA of 50 and 100 p.p.m. induced earlier flowering and increased the number of seed pods set during the experiment. Both protein and total nitrogen of leaves decreased with increasing Na_2EDTA concentration. Analyses of leaf tissues indicate that EDTA was absorbed by the plants. Oxygen uptake of leaf discs was not affected greatly up to 150 p.p.m. of Na_2EDTA , at which point respiration increased. With increasing concentration of Na_2EDTA , cytochrome oxidase and ascorbic acid oxidase activities decreased, while polyphenol oxidase increased. Summation of total oxygen uptake for these three terminal oxidases results in remarkable uniformity up to 150 p.p.m. At 200 p.p.m., there is an increase in these values which parallels the increase in oxygen uptake. These results suggest that under adverse conditions, the respiratory pathway can shift from one to another of these terminal oxidases, with over-all respiration remaining relatively constant. Results of certain other enzyme determinations do not exhibit clear-cut associations with metal requirements or functions.

INTRODUCTION

The mode of action of naturally occurring and synthetic chelating agents in plant nutrition and metabolism is little known, but their capacity to complex metals in soluble or insoluble forms is of great interest in plant physiology and biochemistry. The most widely known of the synthetic chelating agents is ethylenediaminetetraacetic acid (EDTA). The ferric salt is extensively used to correct iron deficiency chlorosis in many plants (23, 24, 30, 31, 38). Absorption of EDTA or its metal chelates has been in question for some time, although there was considerable indirect and in-

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conclusive evidence that the molecule was absorbed (30, 36). Recently Wallace *et al.* (32) presented direct conclusive evidence of EDTA absorption by roots of plants grown both in soils and in culture solutions.

Several interesting phenomena have been attributed to the action of EDTA by *in vitro* studies. Gross (7) and Bowen and Kerwin (3) have reported that EDTA, depending on concentration, may inhibit or stimulate activity of Ca^{++} -activated myosin adenosinetriphosphatase. Webster (34) reported inhibition of Mg^{++} -activated muscle adenylate kinase by EDTA, presumably by the formation of Mg -EDTA chelate, thereby inactivating Mg^{++} ions. Several investigators have found that EDTA stimulates the activity of certain enzymes due to removal of traces of inhibitory heavy metals (1, 9, 13, 20, 25). The unusual chelating properties of EDTA lead one to believe that, in most cases at least, EDTA in the proper concentration will inhibit activity of heavy metal-activated enzymes where the metal-enzyme combination is loosely bound.

Other reports, possibly related to these inhibitory or stimulatory effects on enzymes, state that EDTA exhibits properties of growth regulation by virtue of the fact that at very low concentrations it will stimulate elongation and water uptake of coleoptile or hypocotyl sections of certain plants (2, p. 286; 8; 35).

In the following experiments, studies were carried out with intact sunflower and soybean plants in order to study EDTA absorption by roots and effects of this material on nitrogenous constituents and on some metal- and nonmetal-containing enzymes.

MATERIALS AND METHODS

The first experiment was begun on August 20, 1953. Sixteen solution cultures were set up, each culture vessel containing three Russian sunflower (*Helianthus annuus* L.) seedlings previously germinated in white quartz sand. Composition of the nutrient solution used was as follows: macronutrient salts— $0.001M$ KH_2PO_4 , $0.005M$ $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, $0.002M$ $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and $0.002M$ K_2SO_4 ; micronutrient elements— 0.50 p.p.m. Fe, 0.25 p.p.m. Mn, 0.10 p.p.m. B, 0.10 p.p.m. Zn, 0.01 p.p.m. Cu, and 0.01 p.p.m. Mo. All nutrient solutions were prepared with distilled water and reagent-grade salts. Nutrient treatments of disodium EDTA (Na_2EDTA) at concentrations of 0, 5, 10, 25, 50, 100, 150, and 200 p.p.m. were started on September 1. These levels of Na_2EDTA constitute a range from the optimum level for supplying iron (molar ratio of iron to EDTA of approximately 1:1) to a toxic level (molar ratio of iron to EDTA of approximately 1:60). Treatments were duplicated and nutrient solutions renewed twice weekly. A final harvest of plants was made on October 5, 1953.

The second experiment was begun on October 18, 1954. Sixteen solu-

tion cultures were set up, each culture vessel containing three Hawkeye soybean (*Glycine max* Merr.) seedlings, previously germinated on filter paper in Petri dishes. Composition of nutrient solution was as previously described with one exception: differential sodium levels introduced by the use of increasing concentrations of Na_2EDTA were equally adjusted by the addition of suitable quantities of Na_2SO_4 . Thus, the only nutrient variables were EDTA and $\text{SO}_4^{=}$ concentrations. Nutrient solutions were formulated from deionized water and reagent-grade salts. Each Na_2EDTA level was duplicated and nutrient solutions were supplied by the continuous renewal method (18, 19). Seedlings were two weeks old when treatment was begun. A final harvest of plants was made on January 20, 1955. Tissues were dried at 60°C . in a forced-draft oven for dry weight determinations and chemical analysis. Dry tissues were ground in a Wiley mill to pass a 60-mesh screen.

Leaf tissues for respiration or enzyme activity measurements were removed from experimental plants after distinct leaf symptoms of EDTA toxicity became apparent on plants grown at high EDTA levels.

EDTA was determined by the dithiooxalate method of Darbey (4). One-gram aliquots of tissue were extracted with 20 ml. of water on a steam bath for one hour. The extract was centrifuged and the supernatant decanted and saved. The residue was re-extracted with 15 ml. and then 10 ml. of water. To the combined supernatants was added sufficient Nuchar C-115-N⁶ to decolorize the amber-colored extract. The mixture was stirred, filtered through Whatman No. 42 filter paper, adjusted to pH 6.5 with NaOH, and made to 50 ml. volume with water. Ten milliliter aliquots were then used for the EDTA analysis.

Total nitrogen was determined by the semi-micro Kjeldahl procedure (16, pp. 805–806). Protein nitrogen of leaf tissues was determined by extracting small samples of dried tissue for 16 hours with a refluxing water-alcohol mixture (29). The residue was analyzed by the semi-micro Kjeldahl procedure. Protein nitrogen of enzyme preparations was determined by extracting aliquots of the enzyme solutions in 30-ml. Kjeldahl flasks with sufficient 95 per cent ethanol to give a final ethanol concentration of 80 per cent. Extraction was carried out on a water bath at 50°C . The extract was centrifuged while hot and the supernatant discarded. The residue was re-extracted twice with 80 per cent ethanol, centrifuged each time, the residue digested, and nitrogen determined by the semi-micro Kjeldahl procedure. Nitrate nitrogen was determined on water extracts of tissues by reduction with iron powder (26) and distillation of ammonia. Free ammonia nitrogen was determined by distillation (28). For amide nitrogen

⁶ Activated charcoal obtained from Industrial Chemical Sales, Division of West Virginia Pulp and Paper Co.

determination, H_2SO_4 was added to a suitable aliquot to give a final acid concentration of 1*N*. The mixture was then hydrolyzed for three hours in a boiling water bath and released ammonia determined by distillation (28). Free α -amino nitrogen was determined by the van Slyke procedure (17, pp. 385-393).

About 3 to 5 grams of leaf tissues were harvested for each series of enzyme activity determinations. Care was exercised to sample leaf tissues of about the same physiological age and relative position on the plants. Mid-veins were removed, the tissues were cut into small pieces, mixed thoroughly, and then aliquoted for grinding. Tissues were ground in an ice-cold Ten Broeck glass homogenizer in ten times their weight of ice-cold *M*/15 phosphate buffer, usually adjusted to the pH at which the assay would be carried out. Whole tissue homogenates were used for lactic dehydrogenase, glycolic dehydrogenase, oxalacetic decarboxylase, and indoleacetic acid oxidase. Cell wall debris obtained by low speed centrifugation was used for ascorbic acid oxidase. Cell-free extracts were prepared by centrifuging homogenates at 15,000 \times gravity for 15 minutes. These preparations were used for catalase, polyphenol oxidase, peroxidase, isocitric dehydrogenase, malic dehydrogenase, and diaphorase. The residue after high speed centrifugation was taken up in cold buffer and used for cytochrome oxidase determinations.

Oxygen uptake of leaf tissues was determined manometrically using the method of Klinker (12). One hundred leaf discs 5 mm. in diameter were cut for each measurement. The discs were washed in deionized water, blotted dry, and 10 discs placed in each Warburg vessel on a moistened pad of filter paper. Determinations were run in duplicate. Protein nitrogen was determined on the remaining discs.

Cytochrome oxidase activity was determined by measuring the rate of oxidation of ferrocytochrome *c* at 550 $\text{m}\mu$ on a Beckman DU spectrophotometer (37). Rate of oxidation was converted to microliters of oxygen uptake according to the procedure outlined by Fritz and Beevers (5).

Catalase activity was assayed manometrically (37). Peroxidase and polyphenol oxidase were measured by following the rate of oxidation of reduced 2,3',6-trichlorobenzeneindophenol spectrophotometrically at 530 $\text{m}\mu$ in the presence of hydrogen peroxide and catechol, respectively (21, 22). Optical density changes due to activity of polyphenol oxidase were converted to microliters of oxygen uptake by using a molecular extinction coefficient of 2.52×10^4 . This value was determined experimentally.

Oxalacetic decarboxylase (27), ascorbic acid oxidase (33), and glycolic and lactic dehydrogenases (15) were determined manometrically.

TPN-isocitric dehydrogenase (14), DPNH-diaphorase (14), and DPN-malic dehydrogenase were determined spectrophotometrically at 340 $\text{m}\mu$. The reaction mixture for malic dehydrogenase consisted of: 2.75 ml. of

0.1 *M* phosphate buffer, pH 7.5; 0.10 ml. of 0.1 *M* $MgCl_2$; 0.02 to 0.10 ml. of cell-free extract; 0.10 ml. of 0.002 *M* DPN; and 0.10 ml. of 0.04 *M* i-malate adjusted to pH 7.5.

Indoleacetic acid oxidase of root tissues was determined according to the method of Galston and Dalberg (6).

RESULTS AND DISCUSSION

Symptoms of EDTA toxicity became evident on sunflower plants within two weeks after initiation of treatments in cultures supplied with 150 and 200 p.p.m. of Na_2EDTA . Upper leaves of these plants exhibited a slight systemic chlorosis. By the third week the chlorosis became more severe and also appeared on plants grown with 100 p.p.m. of Na_2EDTA . The toxicity pattern most resembled the chlorosis induced by low calcium or low boron. Lower leaves became bronzed and finally abscised. Oxygen

TABLE I
GROWTH RESPONSES OF HAWKEYE SOYBEAN PLANTS GROWN IN CULTURE SOLUTIONS
WITH DIFFERENT NUTRIENT LEVELS OF Na_2EDTA

| Concentration of Na ₂ EDTA (p.p.m.) | Av. height of plants (cm.) | Av. No. of pods/culture | Av. size of leaves* | | Length/width ratio |
|--|----------------------------------|----------------------------|---------------------|----------------|-----------------------|
| | | | Length (mm.) | Width (mm.) | |
| 0 | 20.5 | 0 | 70 | 47 | 1.50 |
| 5 | 23.7 | 0.5 | 85 | 58 | 1.46 |
| 10 | 25.7 | 1.0 | 87 | 61 | 1.42 |
| 25 | 22.7 | 1.0 | 87 | 63 | 1.39 |
| 50 | 23.5 | 3.5 | 83 | 59 | 1.42 |
| 100 | 22.8 | 3.5 | 78 | 57 | 1.39 |
| 150 | 18.3 | 0 | 76 | 55 | 1.40 |
| 200 | 18.0 | 0 | 73 | 50 | 1.48 |

* Deviation required for significance at 0.01: length=0.95, width=0.94.

uptake decreased in leaf discs from plants growing in nutrient solutions containing increasing concentrations of Na_2EDTA . Assuming no inhibition of oxygen uptake at 0 p.p.m. of Na_2EDTA , it was found that 50 p.p.m. resulted in 9 per cent, 100 p.p.m. in 30 per cent, and 200 p.p.m. in 40 per cent inhibition.

In the soybean experiment, toxicity symptoms were distinct at the 150 and 200 p.p.m. levels within three weeks after the experiment began. Upper leaflets were diffusely mottled and became very stiff. Lower leaflets exhibited interveinal bronzing. The most striking symptom associated with high Na_2EDTA levels was a severe twisting of the leaflets on the upper portion of the plant. This phenomenon appeared after five weeks of treatment. Each leaflet of the trifoliate leaf twisted through angles ranging from 90 to 270 degrees. This was also accompanied by an increase in the angle between the petiole and the stem. Severity of these symptoms was associated directly with Na_2EDTA levels of 25, 50, 100, 150, and 200

p.p.m. Root development was fair at 0 p.p.m., excellent at 5, 10, 25, and 50 p.p.m., and became progressively poorer at 100, 150, and 200 p.p.m. of Na_2EDTA .

Growth data after seven weeks of treatment are shown in Table I. Figure 1 shows a general view of typical growth response after 30 days. In Figure 2, twisting of leaflets at 200 p.p.m. of Na_2EDTA after 35 days is evident.

Average final harvest values are given in Table II. No values are given for the plants grown at the 150 and 200 p.p.m. levels, since these plants died before the final harvest.

Data in Tables I and II show that maximum height and fresh weight yield were attained at 10 p.p.m. of Na_2EDTA . Maximum dry weight yield



FIGURE 1. Hawkeye soybean plants grown in solution cultures supplied with different nutrient concentrations of Na_2EDTA , left to right: (A) 0, 5, 10, 25, and (B) 50, 100, 150, 200 p.p.m. Photograph taken 30 days after initiation of nutrient treatments.

was obtained at 5 p.p.m. When no Na_2EDTA was supplied, fresh and dry weight yields were only about one-third as much as those of all other treatments. Although results in Table I indicate that Na_2EDTA treat-



FIGURE 2. Twisting of leaflets of Hawkeye soybean plants grown with a nutrient concentration of 200 p.p.m. of Na_2EDTA . Photograph taken 35 days after initiation of nutrient treatments.

TABLE II
AVERAGE FRESH WEIGHT AND DRY WEIGHT VALUES OF TISSUE FRACTIONS
OF HAWKEYE SOYBEAN PLANTS GROWN WITH DIFFERENT
NUTRIENT CONCENTRATIONS OF Na_2EDTA

| Concentration of Na_2EDTA (p.p.m.) | Plant fraction | Av. fresh wt./culture (g.) | Av. dry wt./culture (g.) | % Dry wt. |
|--|-------------------|----------------------------------|--------------------------------|--------------|
| 0 | Leaves | 18.4 | 3.10 | 16.8 |
| | Stems | 20.0 | 3.56 | 17.8 |
| | Roots | 27.5 | 1.91 | 6.9 |
| | Pods | — | — | — |
| | | 65.9 | 8.57 | |
| 5 | Leaves | 59.9 | 16.40 | 27.4 |
| | Stems | 40.2 | 7.81 | 19.4 |
| | Roots | 103.8 | 6.81 | 6.6 |
| | Pods | 0.7 | 0.15 | 21.4 |
| | | 204.6 | 31.17 | |
| 10 | Leaves | 55.0 | 15.43 | 28.0 |
| | Stems | 39.3 | 7.49 | 19.0 |
| | Roots | 113.2 | 6.41 | 5.7 |
| | Pods | 0.7 | 0.12 | 17.2 |
| | | 208.2 | 29.45 | |
| 25 | Leaves | 51.1 | 14.10 | 27.6 |
| | Stems | 40.4 | 7.21 | 17.9 |
| | Roots | 104.7 | 5.79 | 5.5 |
| | Pods | 0.6 | 0.20 | 33.3 |
| | | 196.8 | 27.30 | |
| 50 | Leaves | 47.1 | 12.81 | 27.2 |
| | Stems | 36.7 | 6.54 | 17.8 |
| | Roots | 121.1 | 7.43 | 6.1 |
| | Pods | 2.3 | 0.48 | 20.9 |
| | | 207.2 | 27.26 | |
| 100 | Leaves | 40.3 | 11.21 | 27.8 |
| | Stems | 34.5 | 5.92 | 17.2 |
| | Roots | 105.2 | 6.47 | 6.1 |
| | Pods | 2.2 | 0.62 | 28.2 |
| | | 182.2 | 24.22 | |

ments significantly affected leaf size, the length-width ratio indicates clearly that there was no effect on leaf shape. Na_2EDTA levels of 50 and 100 p.p.m. induced earlier flowering and increased the number of seed pods set during the course of the experiment.

Results of determinations of nitrogenous constituents are given in Table III. These data indicate clearly that both total and protein nitrogen of leaf tissues decrease with increasing Na_2EDTA concentration. Nitrate, ammonia, amide, free α -amino nitrogen do not show any clear-cut dif-

ferences with the exception of a possible accumulation of nitrate, amide, and free α -amino nitrogen in tissue receiving no EDTA. It is clear that the major changes brought about by EDTA accumulation are manifested primarily in the total and protein nitrogen fractions. Proportion of protein nitrogen in total nitrogen increased 20 per cent in plants treated with from 0 to 10 p.p.m. EDTA, decreased slightly at 25 to 100 p.p.m., and dropped to control level in tissues showing severe toxicity symptoms (150 and 200 p.p.m.).

These observations can be interpreted to mean that if EDTA restricted uptake of nitrogen by the plants, the requirements for growth caused

TABLE III

NITROGENOUS COMPONENTS OF TISSUE FRACTIONS OF LEAF TISSUES OF HAWKEYE SOYBEAN PLANTS GROWN WITH DIFFERENT NUTRIENT CONCENTRATIONS OF Na_2EDTA

| Concentration of Na_2EDTA (p.p.m.) | Per cent of dry weight | | | | | | Protein N as % of Total N |
|--|------------------------|-----------|-----------|-----------|---------|------------------------|---------------------------|
| | Total N | Protein N | Nitrate N | Ammonia N | Amide N | Free α -Amino N | |
| 0 | 4.44 | 2.41 | 0.55 | 0.028 | 0.32 | 0.46 | 54 |
| 5 | 3.75 | 2.29 | 0.30 | 0.026 | 0.21 | 0.35 | 61 |
| 10 | 3.63 | 2.24 | 0.28 | 0.026 | 0.20 | 0.33 | 64 |
| 25 | 3.72 | 2.18 | 0.28 | 0.031 | 0.21 | 0.37 | 59 |
| 50 | 3.70 | 2.26 | 0.30 | 0.021 | 0.28 | 0.36 | 61 |
| 100 | 3.42 | 2.09 | 0.27 | 0.023 | 0.20 | 0.34 | 61 |
| 150 | 3.02 | 1.65 | 0.32 | 0.025 | 0.22 | 0.35 | 55 |
| 200 | 2.40 | 1.32 | 0.31 | 0.027 | 0.25 | 0.30 | 55 |

greater utilization of total leaf nitrogen for protein formation where growth was enhanced by EDTA. The decreases in proportion of protein nitrogen in concentrations of 25 p.p.m. or more presumably reflect the toxicity of EDTA to the leaf tissues. Since free α -amino nitrogen does not accumulate as protein nitrogen falls off, EDTA is apparently not inducing rapid proteolysis. At the same time, there is no question that EDTA is being absorbed by these plants and translocated into the leaves as can be seen in Figure 3. This would tend to confirm suggestions that EDTA is poorly metabolized by biological systems (10).

Enzyme data in Tables IV and V show distinct but aberrant patterns. These patterns do not necessarily appear to be associated with any metal requirements of these enzymes.

Oxygen uptake of leaf discs shows little difference up to 150 p.p.m. of Na_2EDTA , at which point it increases sharply. These results differ considerably from those obtained in the sunflower experiment where the sodium content was varied according to the amount of Na_2EDTA supplied. In the soybean experiment sodium supply was held constant by addition of Na_2SO_4 .

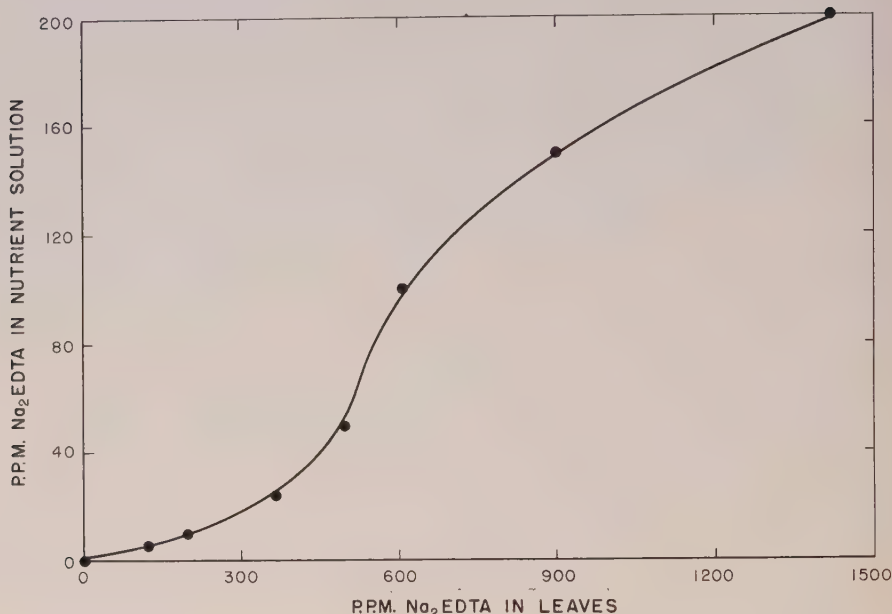


FIGURE 3. Accumulation of Na_2EDTA in leaflets of Hawkeye soybean plants grown with different nutrient concentrations of Na_2EDTA .

Noteworthy among the data of enzyme activities are those for polyphenol oxidase and cytochrome oxidase. With nutrient concentrations of Na_2EDTA up to 100 p.p.m., the activities of these enzymes show an inverse relationship. Above 100 p.p.m., where actual toxic symptoms were noted on leaves, both enzymes increase considerably in activity as do most of the enzymes assayed. Ascorbic acid oxidase exhibits a continuous slight decrease in activity up to 100 p.p.m., then drops sharply at 150 p.p.m. If a summation is made of the total oxygen uptake by the three systems which are known to act as terminal oxidases (in some plant tissues at least; this

TABLE IV
EFFECT OF DIFFERENT NUTRIENT CONCENTRATIONS OF Na_2EDTA ON ACTIVITIES OF HAWKEYE SOYBEAN LEAF TERMINAL OXIDASES. VALUES EXPRESSED AS MICROLITERS OF OXYGEN UPTAKE PER HOUR PER MILLIGRAM OF PROTEIN NITROGEN

| | Nutrient concn. of Na_2EDTA p.p.m. | | | | | | | |
|-----------------------|--|------|------|------|------|------|------|------|
| | 0 | 5 | 10 | 25 | 50 | 100 | 150 | 200 |
| Cytochrome oxidase | 570 | 553 | 416 | 411 | 408 | 370 | 586 | 874 |
| Polyphenol oxidase | 495 | 477 | 597 | 601 | 606 | 766 | 1151 | 1863 |
| Ascorbic acid oxidase | 1535 | 1366 | 1410 | 1458 | 1270 | 1203 | 442 | — |
| Total | 2510 | 2396 | 2423 | 2470 | 2284 | 2339 | 2179 | 2737 |

point has not been proved for soybean leaf respiration) a remarkable uniformity is found through 150 p.p.m. The increase evident at 200 p.p.m. parallels the increase in over-all oxygen uptake; this is probably a consequence of the extreme toxicity of EDTA at this concentration.

In the study of plant respiration it has on occasion seemed desirable to speculate that several terminal oxidases may co-exist in the tissues, only one of which may mediate the bulk of terminal respiration under normal conditions (11, pp. 216–228). Under a drastic change from normal physiological conditions it might be postulated that the respiration pathway could shift from one to another of these terminal oxidases while the total oxygen uptake remains relatively constant. These data on cytochrome oxidase, polyphenol oxidase, and ascorbic acid oxidase are in accord with such a speculation, and suggest the desirability of further study of plant respiration from this point of view. It must be kept in mind, however, that the methods of assay employed do not necessarily prove participation of these enzymes in normal respiratory mechanisms, but merely establish their presence in the leaf tissues.

The action of EDTA in bringing about a decrease in cytochrome oxidase activity may be due to successful competition by EDTA with porphyrin moieties for iron, resulting in decreased synthesis of the enzyme or to direct inhibition of protein synthesis. The concomitant increase in polyphenol oxidase may be due to the shifting of a portion of terminal oxidation under conditions of low cytochrome oxidase activity. On the other hand, the heme enzyme, catalase, increases in activity over most of the range of Na_2EDTA concentrations that inhibit cytochrome oxidase. Peroxidase activity is high at 0 p.p.m. of Na_2EDTA but then drops to a fairly constant level of activity up to 100 p.p.m. The action of EDTA on activities of these structurally related enzymes presents an ambiguous picture and suggests that EDTA exerts its effect in part, at least, by both qualitatively and quantitatively inhibiting formation of certain proteins.

At this point no specific interpretations can be offered of the remaining enzyme patterns shown in Table V. It is possible that with increasing supply of EDTA in the substrate, the concentration attained in the tissues would reach a level high enough to complex most of the free or loosely bound heavy metals, and would consequently have drastic effects on activities of a great many enzymes. The complexity of the situation obviously is such that no simple explanations or interpretations can be attempted.

This work was carried out with grants-in-aid from Versenes Incorporated, Dow Chemical Company, Framingham, Mass., Refined Products Corp., Lyndhurst, N. J., Niagara Chemical Division, Food Machinery and Chemical Corp., Middleport, N. Y., and in part by the Frascch Foundation for Agricultural Chemistry.

TABLE V

EFFECT OF DIFFERENT NUTRIENT CONCENTRATIONS OF Na_2EDTA ON ACTIVITIES OF HAWKEYE SOYBEAN LEAF ENZYMES. VALUES EXPRESSED ON A MILLIGRAM OF PROTEIN NITROGEN BASIS

| | Nutrient concentration of Na_2EDTA (p.p.m.) | | | | | | | |
|--|---|--------|--------|--------|--------|--------|--------|--------|
| | 0 | 5 | 10 | 25 | 50 | 100 | 150 | 200 |
| Respiration $\mu\text{l. O}_2$ uptake/hr. | 210 | 170 | — | 182 | 171 | 186 | 245 | 389 |
| Catalase $\mu\text{l. O}_2$ evolved/min. | 5,230 | 17,830 | 25,200 | 27,450 | 22,250 | 21,500 | 14,750 | 14,100 |
| Peroxidase Δ Optical density increase $\times 1000/\text{min.}$ | 16,560 | 3,700 | 3,620 | 3,460 | 3,400 | 3,610 | 6,740 | 14,100 |
| Oxalacetic decarboxylase $\mu\text{l. CO}_2$ evolved/hr. | 207 | 167 | — | 139 | 105 | 79 | — | 256 |
| Lactic dehydrogenase $\mu\text{l. O}_2$ uptake/hr. | 357 | 523 | — | 355 | 381 | 359 | — | 145 |
| Glycolic dehydrogenase $\mu\text{l. O}_2$ uptake/hr. | 260 | 313 | — | 187 | 198 | 208 | — | 9 |
| TPN-Isocitric dehydrogenase Δ Optical density increase $\times 1000/\text{min.}$ | 397 | 517 | — | 560 | 566 | 715 | — | 900 |
| DPN-Malic dehydrogenase Δ Optical density increase $\times 1000/\text{min.}$ | 298 | 302 | — | 435 | 396 | 358 | — | 655 |
| DPNH-Diaphorase Δ Optical density decrease $\times 1000/\text{min.}$ | 2,850 | 4,050 | — | 5,110 | 5,100 | 5,120 | — | 6,400 |
| Indoleacetic acid oxidase* $\mu\text{g. IAA}$ oxidized/hr. | 8 | 8 | 31 | 14 | 7 | 10 | 73 | — |

* Determinations of indoleacetic acid oxidase were made on root tissues.

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AMINO ACID COMPOSITION OF SOUTHERN BEAN MOSAIC VIRUS

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SUMMARY

The amino acid composition of a single preparation of southern bean mosaic virus was estimated by paper chromatography. These values are compared with those reported for tobacco mosaic virus, turnip yellow mosaic virus and tomato bushy stunt virus and fall within the same range, with the exception that the tyrosine content is higher and the valine lower than in the other three viruses.

The approximate molecular weight of a protein sub-unit of southern bean mosaic virus is 27,000, or an integral number thereof, when calculated from the quantity of cystine present.

As part of a program on the structure of southern bean mosaic virus (SBMV), the amino acid composition of the protein component of the virus was determined. From such information the minimum molecular weight of the protein sub-unit, a necessary supplement to the general program, can be derived.

Of the plant viruses, only tobacco mosaic virus (TMV) and its many strains, tomato bushy stunt virus (TBSV) and turnip yellow mosaic virus (TYMV) have been analyzed thus far for amino acids. It is of interest to know the composition of other plant viruses in order to compare them. Such comparisons will point out similarities and differences in their amino acid compositions which may eventually help in classifying the viruses as related either to each other or to their respective hosts.

EXPERIMENTAL

Purification of southern bean mosaic virus. Bean plants (*Phaseolus vulgaris* L. var. Bountiful) grown in the field were inoculated with SBMV and were harvested three weeks later.

The purification procedure was an established one used for plant viruses. After clarification of the sap with 20 per cent absolute alcohol, the virus was precipitated with $1/3$ saturated ammonium sulfate. Although the salting out was repeated 4 times, the solution still remained highly pigmented and contained low molecular weight components. The pigment was removed by adsorption of the virus on a strongly basic exchange resin (Rohm and Haas, Amberlite, XE-67), according to Shainoff and Lauffer (10), and the virus was then eluted with chloride-phosphate buffer at pH 6.85, ionic strength 0.08. The SBMV and the low molecular weight contaminants were separated by differential centrifugation in the Spinco preparative ultra-centrifuge Model L.

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From the moistened pellet, colorless virus crystals were formed (7), re-dissolved and checked for purity. A single boundary in both the ascending and descending limbs of a Tiselius cell indicated the presence of a single component. A sedimentation constant of 114 S was obtained and is that of SBMV (5). To establish infectivity of the virus, pinto beans were inoculated and local lesions developed.

Chromatographic procedures. The solution of the redissolved crystals was dialyzed against distilled water and then dried by lyophilization. Approximately 22 mg. of the lyophilized product, containing 9 per cent moisture, were hydrolyzed with 20 ml. of 6*N* HCl under reflux for 20 hours. The pink color in the early stages of hydrolysis suggests the presence of tryptophan in the virus. At the end of the hydrolysis, the HCl was removed and the residue taken up in 1 ml. of 10 per cent 2-propanol (1). The N content of this solution was 2.75 mg., equivalent to 16.2 mg. of virus, on the assumption that it contained 17 per cent N (5).

The amino acid composition of the hydrolyzate was estimated by paper chromatography densitometrically using 4 or 5 replicate spots for each amino acid at 3 or 4 levels of protein hydrolyzate. Previous experience (1) has shown that the average reproducibility of duplicate analyses by this procedure is approximately ± 10 per cent.

RESULTS AND DISCUSSION

Table I gives the amino acid composition of SBMV in grams of residue per 100 grams of virus, the total nitrogen and the molar ratios to the nearest whole number as calculated from the composition. The listed

TABLE I
AMINO ACID COMPOSITION OF SOUTHERN BEAN MOSAIC VIRUS

| | G. residue 100 g. virus | G. amino acid N 100 g. proten | Molar ratios (whole numbers) |
|---------------|----------------------------|----------------------------------|---------------------------------|
| Alanine | 3.7 | 0.92 | 18 |
| Arginine | 6.8 | 3.12 | 15 |
| Aspartic acid | 5.8 | 0.89 | 17 |
| Cystine | 0.6 | 0.11 | 1 |
| Glutamic acid | 6.0 | 0.84 | 16 |
| Glycine | 3.5 | 1.09 | 21 |
| Histidine | 1.2 | 0.47 | 3 |
| Isoleucine | 4.8 | 0.76 | 14 |
| Leucine | 6.5 | 1.02 | 20 |
| Lysine | 2.7 | 0.74 | 7 |
| Methionine | 2.4 | 0.32 | 6 |
| Phenylalanine | 3.6 | 0.43 | 8 |
| Proline | 3.1 | 0.61 | 11 |
| Serine | 5.3 | 1.07 | 21 |
| Threonine | 8.1 | 1.52 | 29 |
| Tyrosine | 4.7 | 0.51 | 10 |
| Valine | 4.6 | 0.82 | 16 |

values do not take into consideration the destruction of certain amino acids during acid hydrolysis. It is generally recognized that besides tryptophan, which is completely destroyed, certain amino acids may be partially destroyed during acid hydrolysis, especially in the presence of carbohydrate (1). The exact extent of the destruction will vary with the conditions of hydrolysis, the relative proportion of acid to protein, the quantity and type of nonprotein impurities and the particular protein. Because of the *lack of material*, none of these factors could be studied in the present instance.

The uncorrected analytical values as given in the first column, Table I, show that only 94 per cent of the virus is accounted for when assigning a value of 21 per cent for the ribonucleic acid (RNA) present (6). If we assume the same losses of serine (13.6 per cent), threonine (6.5 per cent) and tyrosine (6.0 per cent), which were found for tomato bushy stunt by De Fremery and Knight (2), the respective values are raised to 6.1 per cent serine, 8.7 per cent threonine and 4.9 per cent tyrosine. Smith and Markham (11) have shown that the purines of RNA may decompose during acid hydrolysis to yield glycine. This correction diminishes the glycine content by 9.0 per cent for tomato bushy stunt virus (2), and thus probably reduces the glycine concentration to 3.2 per cent for SBMV. The total recovery becomes 98 per cent when the above corrections are made and upon the assumption that tryptophan is present at 1 per cent concentration and amide nitrogen at 1.2 per cent. These latter values are the averages of those obtained for tobacco mosaic virus (4), turnip yellow mosaic virus (8) and tomato bushy stunt virus (2).

Column 2, Table I, lists the total amino acid nitrogen on the basis of 100 grams of protein. The total nitrogen is 15.3 grams per 100 grams of protein, but by making the appropriate corrections for the amino acid composition, as discussed above, and assuming 1.2 grams of amide nitrogen the total N becomes 16.4 grams. Although this value is somewhat high, nevertheless it is well within the estimated errors of analysis.

Comparison of the amino acid patterns of plant viruses is given in Table II. Because of the different amounts of RNA in each of the viruses, the compositions of TMV (common), TYMV, TBSV and SBMV are given as grams of residue per 100 grams of protein rather than per 100 grams of virus. As can be seen, the compositions vary considerably, but there are certain striking similarities and differences. All of the viruses contain large amounts of leucine and threonine and small amounts of cystine, or cysteine, histidine and methionine. Although the common strain of TMV contains no methionine, Holmes rib-grass strain has 1.9 per cent methionine. TYMV differs markedly from the other viruses in that it has a large percentage of proline and the ratio of arginine to lysine is small, whereas for the other viruses the ratio is large. Both TMV and

TBSV contain larger amounts of aspartic acid than either TYMV or SBMV. In the case of SBMV the content of tyrosine is higher and that of valine lower than in any of the other viruses.

From the amino acid composition it is possible to calculate the minimum molecular weight of the protein sub-unit by assuming one residue per protein chain of the amino acid present in the smallest amount. Using the cysteine content of TMV, a unit of 18,000 molecular weight is calculated. This value has been confirmed by end-group analysis (3, 9). The molar ratio of tryptophan to cysteine, methionine and histidine in TBSV is 1:2:2:3, thus giving approximately 27,000 molecular weight for the

TABLE II
COMPARISON OF AMINO ACID COMPOSITIONS OF SOME PLANT VIRUSES
IN GRAMS OF RESIDUE/100 GRAMS PROTEIN

| | Tobacco mosaic virus (TMV)(4) | Turnip yellow mosaic virus (TYMV)(7) | Tomato bushy stunt virus (TBSV)(2,4) | Southern bean mosaic virus (SBMV) |
|---------------|-------------------------------------|--|--|---|
| Alanine | 4.4 | 4.54 | 5.6 | 4.6 |
| Arginine | 9.4 | 2.24 | 6.8 | 8.6 |
| Aspartic acid | 12.5 | 4.41 | 11.0 | 7.3 |
| Cystine | 0.0 | 2.31 | | 0.8 |
| Cysteine | 0.6 | | 0.7 | |
| Glutamic acid | 10.5 | 8.41 | 6.0 | 7.6 |
| Glycine | 1.5 | 2.20 | 4.8 | 4.4 |
| Histidine | 0.0 | 1.86 | 1.4 | 1.5 |
| Isoleucine | 6.1 | 9.31 | 3.2 | 6.1 |
| Leucine | 8.5 | 8.89 | 10.7 | 8.2 |
| Lysine | 1.4 | 9.40 | 3.7 | 3.4 |
| Methionine | 0.0 | 2.40 | 0.8 | 3.0 |
| Phenylalanine | 8.0 | 3.39 | 4.4 | 4.6 |
| Proline | 5.2 | 9.04 | 3.3 | 3.9 |
| Serine | 6.4 | 6.71 | 6.7 | 6.7 |
| Threonine | 9.0 | 12.80 | 9.7 | 10.2 |
| Tryptophan | 2.0 | 1.04 | 0.7 | |
| Tyrosine | 3.6 | 2.20 | 3.7 | 5.9 |
| Valine | 8.3 | 7.14 | 8.7 | 5.8 |

sub-unit. Similar calculations for TYMV, assuming 2 tryptophan, 4 cystine, 5 arginine, 5 histidine and 5 tyrosine residues per protein chain, show that the sub-unit is of molecular weight about 36,000. With a molar ratio of cystine to histidine of 1:3, the molecular weight of the protein chain in SBMV is 26,000 to 27,000. The size of the sub-unit thus calculated for SBMV is of the same order of magnitude as those given above for other viruses, although confirmation will have to await further analysis.

The authors wish to thank Dr. D. L. D. Casper, Biophysics Dept., Yale University, New Haven, Conn., for determining the sedimentation constant, and James Beaudry for assaying the nitrogen content.

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FUNGITOXICITY OF SUBSTITUTED *s*-TRIAZINES

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SUMMARY

A new class of highly effective fungicides has been created by reacting cyanuric chloride with compounds having an active hydrogen in the presence of a base. The three chlorine atoms of cyanuric chloride were replaced stepwise with arylamino, aryloxy, alkoxy, and alkylamino groups to give mono, di, and trisubstituted symmetrical triazines by controlling the concentrations and conditions of reaction. Some 70 compounds and 8 other reaction products were evaluated for ability to prevent germination of spores of *Alternaria oleracea* Milb. and *Monilinia fructicola* (Wint.) Honey on glass slides, infection of tomato foliage by *Alternaria solani* (Ell. & Mart.) Jones & Grout and *Phytophthora infestans* (Mont.) DeBary, pea seed decay by *Pythium* sp. and other soil inhabiting fungi and fabric deterioration by cellulose-destroying microorganisms.

Although the compounds performed somewhat differently in the several tests, the following generalizations may be made. The substitution of a single arylamino or aryloxy group yielded highly fungitoxic dichlorotriazines. The corresponding disubstituted analogues were much less effective while trisubstituted analogues were essentially ineffective. The arylamino dichloro-*s*-triazines were more effective fungicides and more stable chemicals than analogous aryloxy compounds.

In the arylamino dichloro-*s*-triazines the substituents on the arylamino group decisively modified the fungitoxicity. Compounds with halogen and methyl groups were more effective than those with nitro, cyano, or phenylazo groups. The *ortho*, chloro, bromo, and methylanilino compounds were very active foliage fungicides while the *p*-chloroanilino compound was more effective as a seed protectant and fabric preservative. The monochloroanilino compounds had a wider spectrum of effectiveness than the monobromo or monoethyl analogues. Compounds containing more than one chlorine on either arylamino or aryloxy groups were less active.

The most promising member of the series was 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine. This material had an ED₅₀ of 0.04 p.p.m. against *A. oleracea* and 1.5 p.p.m. against *M. fructicola* in spore inhibition tests and an ED₉₅ of 80 p.p.m. against both *A. solani* and *P. infestans* on tomato foliage. Cotton fabric was preserved at a concentration of 1.0 per cent. This performance is equal or superior to commercially successful materials now in use. It was somewhat less effective in preventing seed decay and damping off.

INTRODUCTION

An imposing number of heterocyclic nitrogen compounds are known to be fungitoxic. Such commercially successful representatives as the

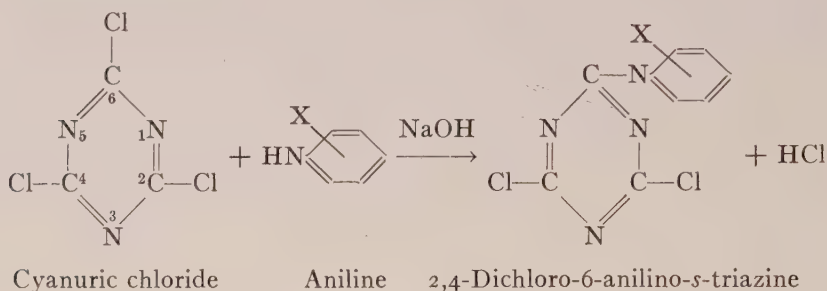
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imidazolines (15) and quinolines (13) and promising experimental materials such as the substituted pyrazoles (11), phenothiazines (5), phthalimides (7), and pyrimidines (12) indicate that fungitoxicity is fairly common. In a study of some 165 heterocyclic nitrogen compounds of many different types, Horsfall and Rich (6) demonstrated by spore inhibition studies that a surprisingly high percentage of these chemicals had fungicidal tendencies.

Certain derivatives of the symmetrical triazines were added to this list as fungicides in 1951. Some arsenical derivatives of *s*-triazines were shown to be trypanocidal and spirochetocidal in 1944 (4). The compounds in this study are essentially derivatives of cyanuric chloride obtained by the following type reaction:



The substituted compounds as described in preliminary reports (16, 17) are more stable and more fungitoxic than cyanuric chloride. The replacement of a chlorine in cyanuric chloride by a compound with active hydrogen in the presence of a base offers many possibilities for creating analogues. Chlorine may be replaced successively to give mono, di, and trisubstituted products. A variety of arylamino, aryloxy, or alkyl compounds can be used as reactants to vary the character of the substituent group and its attachment to the basic nucleus. Approximately 80 compounds were synthesized and tested as fungicides during the period 1951-1954. Data are presented in this paper on 62 of these compounds which showed fungicidal tendencies and the relationship of chemical structure to activity is discussed.

METHODS OF SYNTHESIS AND PHYSICAL PROPERTIES OF SUBSTITUTED *s*-TRIAZINES

The triazines were synthesized by the reaction indicated above but the details varied somewhat depending upon whether (a) alkylamino- or arylamino-*s*-triazines, (b) halogenated dichloro-aryl-amino-*s*-triazines, or (c) alkoxy- or aryloxy-*s*-triazines were being prepared.

Alkylamino- and arylamino-s-triazines. Two procedures were used for synthesizing these compounds. In the first procedure a solution of one mole of cyanuric chloride in acetone was added slowly to ice water to ob-

tain a fine suspension. To the vigorously stirred mixture was added dropwise one mole of the amine or an acetone solution of the amine, followed by an aqueous solution of one equivalent of sodium hydroxide or sodium carbonate. The reaction temperature was maintained at 0° to 5° C. throughout the additions and was stirred for one to two hours until the mixture reached room temperature. The solid material was collected on a suction filter washed with water, dried in air and recrystallized from a suitable solvent. This method is preferred over the second procedure because an inorganic base is used and an acid wash of the crude product is not required.

By the second method, one mole of cyanuric chloride in benzene was cooled to 0° to 10° C. Two moles of the amine in benzene were added dropwise with vigorous stirring. The mixture was stirred for one to two hours until room temperature was reached. The solid which precipitated was collected on a suction filter, washed successively with dilute hydrochloric acid and water, and dried. Concentration of the benzene filtrate by evaporation yielded an additional quantity of crystalline product. Only two members of the group were prepared by this method.

Twenty-five, 2,4-dichloro-6-arylamino-*s*-triazines and two 2,4-dichloro-6-alkylamino-*s*-triazines are listed in Table I with their physical properties.

The diaminomonochloro-*s*-triazines listed in Table I were prepared in a similar manner by slowly adding two moles of amine to a fine slurry of one mole of cyanuric chloride in ice water. Stirring was continued for 0.5 to 1 hour at 0° to 5° C., then an aqueous solution of two equivalents of sodium hydroxide, sodium carbonate or sodium bicarbonate were added slowly, while the temperature was allowed to rise to 45° to 50° C. and held for one to two hours. After cooling, the solid material was collected on a suction filter, washed with water, dried and purified by recrystallization.

Halogenation of 2,4-dichloro-6-arylamino-s-triazines. The high fungitoxicity of chlorinated and brominated anilino-dichloro-*s*-triazines, namely, 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine, 2,4-dichloro-6-(*p*-chloroanilino)-*s*-triazine, and 2,4-dichloro-6-(*o*-bromoanilino)-*s*-triazine led to experiments in the preparation of structurally similar compounds by direct halogenation of the benzene rings of 2,4-dichloro-6-anilino-*s*-triazine and 2,4-dichloro-6-(*o*-toluidino)-*s*-triazine. A solution of 2,4-dichloro-6-anilino-*s*-triazine or 2,4-dichloro-6-(*o*-toluidino)-*s*-triazine in benzene or carbon tetrachloride was gently refluxed and stirred while dry chlorine or bromine was introduced slowly either in the presence or absence of a catalyst. After cooling, the solid precipitate was collected on a filter, washed with solvent and dried. Table II lists the products obtained.

All the products probably were mixtures as indicated by their wide

TABLE I
 AMINO-S-TRIAZINES SYNTHESIZED FOR EVALUATION AS FUNGICIDES

| Substituents | Crystd. from* | M.p. °C. | Lit. value m.p. °C. | Empirical formula | Analysis, % chlorine | |
|---|---------------|-------------|---------------------|--|----------------------|-------|
| | | | | | Calcd. | Found |
| 2,4-Dichloro-6-(arylamino or alkylamino)-s-triazines | | | | | | |
| Aniline | E | 134-136 | 138 | C ₉ H ₆ N ₄ Cl ₂ | | |
| N-Methylanilino | B | 131-132 | 131-132 | C ₁₀ H ₈ N ₄ Cl ₂ | | |
| o-Toluidino | B | 156-157.5 | | C ₁₀ H ₈ N ₄ Cl ₂ | 27.8 | 28.1 |
| p-Toluidino | B | 130-131 | | C ₁₀ H ₈ N ₄ Cl ₂ | 27.8 | 27.5 |
| o-Ethylanilino | F | 119.5-120.5 | | C ₁₁ H ₁₀ N ₄ Cl ₂ | 26.3 | 26.8 |
| o-Methoxyanilino | — | 174.5-176 | | C ₁₀ H ₈ N ₄ OCl ₂ | | |
| p-Methoxyanilino** | — | 167-169.5 | 168-170 | C ₁₀ H ₈ ON ₄ Cl ₂ | | |
| o-Chloroanilino | E | 155-157 | | C ₉ H ₅ N ₄ Cl ₃ | 38.6 | 38.1 |
| m-Chloroanilino | G | 129-131 | | C ₉ H ₅ N ₄ Cl ₃ | 38.6 | 38.6 |
| p-Chloroanilino** | — | 183-185 | 185-186 | C ₉ H ₅ N ₄ Cl ₃ | | |
| 2,4-Dichloroanilino | B | 149-150.5 | | C ₉ H ₄ N ₄ Cl ₄ | 45.8 | 46.3 |
| 2,5-Dichloroanilino | G | 167-169.5 | | C ₉ H ₄ N ₄ Cl ₄ | 45.8 | 45.8 |
| 3-Chloro-2-methylanilino | B | 196.5-198.5 | | C ₁₀ H ₇ N ₄ Cl ₃ | 36.7 | 37.1 |
| 5-Chloro-2-methylanilino | B | 195-196.5 | | C ₁₀ H ₇ N ₄ Cl ₃ | 36.7 | 37.4 |
| o-Bromoanilino | B | 158.5-159 | | C ₉ H ₅ N ₄ Cl ₂ Br | 22.2 | 22.0 |
| m-Bromoanilino | B | 141.5-142 | | C ₉ H ₅ N ₄ Cl ₂ Br | 22.2 | 21.8 |
| p-Bromoanilino | B | 180-182.5 | | C ₉ H ₅ N ₄ Cl ₂ Br | 22.2 | 21.7 |
| p-Cyanoanilino | C | >360 | | C ₁₀ H ₅ N ₅ Cl ₂ | 26.6 | 26.7 |
| p-Nitroanilino | C | >360 | | C ₉ H ₅ O ₂ N ₅ | 24.8 | 23.7 |
| p-Phenylazaniilino | B | 211-213 | | C ₁₅ H ₁₀ N ₆ Cl ₂ | 20.5 | 20.3 |
| α-Naphthylamino | — | 144-148 | 149 | C ₁₃ H ₈ N ₄ Cl ₂ | | |
| β-Naphthylamino | B | 156.7-158 | 154 | C ₁₃ H ₈ N ₄ Cl ₂ | | |
| o-Phenylanilino | E | 139-141 | | C ₁₅ H ₁₀ N ₄ Cl ₂ | 22.4 | 22.3 |
| p-Phenylanilino | B | 169-172 | | C ₁₅ H ₁₀ N ₄ Cl ₂ | 22.4 | 22.2 |
| N-Ethylanilino | F | 119.5-120.5 | | C ₁₁ H ₁₀ N ₄ Cl ₂ | 26.3 | 26.8 |
| Diisopropylamino | B | 100-103 | | C ₉ H ₁₄ N ₄ Cl ₂ | 28.5 | 28.8 |
| n-Butylamino | B | 49-52 | 51-52 | C ₇ H ₁₀ N ₄ Cl ₂ | | |
| 2-Chloro-4,6-bis(arylamino or alkylamino)-s-triazines | | | | | | |
| Bisanilino | B | 194-195 | 199-201 | C ₁₅ H ₁₂ N ₆ Cl | 11.9 | 11.5 |
| Bis(N-methylanilino) | — | 84.5-87 | | C ₁₇ H ₁₆ N ₆ Cl | 10.9 | 10.8 |
| Bis(p-chloroanilino) | B | 218-219 | 223 | C ₁₅ H ₁₀ N ₆ Cl ₃ | | |
| Bis(2,5-dichloroanilino) | — | 192-194 | | C ₁₅ H ₈ N ₆ Cl ₅ | 40.7 | 41.3 |
| Bis(p-methoxyanilino) | A | 200-201 | 197-199 | C ₁₇ H ₁₆ N ₆ Cl | | |
| Bis(p-nitroanilino) | — | 382-383 | | C ₁₅ H ₁₀ O ₄ N ₇ Cl | 9.2 | 10.2 |
| Bis(α-naphthylamino) | B | 209-210 | 215 | C ₂₃ H ₁₆ N ₆ Cl | 8.9 | 8.9 |
| Bis(n-butylamino) | D | 209-211 | | C ₁₁ H ₂₀ N ₆ Cl | 14.6 | 14.4 |

* Code for solvents used in recrystallization: A=acetone; B=benzene; C=cyclohexanone; D=dioxane; E=benzene-hexane; F=hexane; G=trichloroethylene.

** Prepared in benzene solution.

melting point ranges, shown in Table II. The use of iodine or anhydrous ferric chloride catalysts generally gave a faster rate of halogenation and facilitated the introduction of a second halogen into the molecule. Infra-red analysis of the products showed that monohalogenation of 2,4-dichloro-6-anilino-*s*-triazine yielded the *para* isomer over the *ortho* isomer in a ratio of about 3:1. The chlorine content of this mixed sample as indicated was found to be 40.6 per cent, while that calculated for the pure 2,4-dichloro-6-monochloroanilino-*s*-triazine compounds is 38.6 per cent.

When two halogens were introduced into a molecule of 2,4-dichloro-6-anilino-*s*-triazine, the product obtained was predominantly 2,4 dichloro-6-(2,4-dichloroanilino)-*s*-triazine. This was shown by infrared analysis and also by a mixed melting point determination. Chlorination of 2,4-dichloro-6-(*o*-toluidino)-*s*-triazine in the presence of anhydrous ferric chloride gave a product which was found to contain 37.5 per cent chlorine. The calculated chlorine content for a 2,4-dichloro-6-(chlorotoluidino)-*s*-triazine is 36.7 per cent. It would appear that this product, the chlorinated-*o*-toluidino sample, was essentially a monochloro derivative, but no

TABLE II
HALOGENATED 2,4-DICHLORO-6-ARYLAMINO-*s*-TRIAZINES SYNTHESIZED
FOR EVALUATION AS FUNGICIDES

| Substituent in the 6 position | Reaction solvent | Catalyst | M.p. °C. | Analysis, % | |
|------------------------------------|---------------------|--------------------------|----------|-------------|---------|
| | | | | Chlorine | Bromine |
| Chlorinated anilino | Benzene | None | 133-141 | 40.6 | |
| Dichlorinated anilino | Benzene | I ₂ | 136-139 | 45.8 | |
| Chlorinated- <i>o</i> -toluidino | Benzene | Anhyd. FeCl ₃ | 147-161 | 37.5 | |
| Monobrominated anilino | CCl ₄ | I ₂ | 162-175 | 15.8 | 34.6 |
| Brominated anilino | CCl ₄ | Anhyd. FeCl ₃ | 176-188 | 13.9 | 39.9 |
| Brominated- <i>o</i> -toluidino | Benzene | Anhyd. FeCl ₃ | 144-153 | 13.0 | 36.2 |
| Brominated <i>N</i> -methylanilino | Benzene | Anhyd. FeCl ₃ | 97-105 | 15.2 | 35.2 |
| Brominated <i>N</i> -ethylanilino | Benzene | Anhyd. FeCl ₃ | 131-144 | 15.2 | 32.1 |

attempt was made to determine the position of the chlorine atom introduced.

Bromination of 2,4-dichloro-6-anilino-*s*-triazine and 2,4-dichloro-6-(*o*-toluidino)-*s*-triazine gave a complex mixture of isomers as indicated by the data in Table II. It was found by hydrolysis of brominated 2,4-dichloro-6-anilino-*s*-triazine that some exchange of bromine and chlorine on the triazine ring had taken place. This fact further complicated the isomeric mixtures obtained by bromination. No attempt was made to separate the isomers or determine the extent of halogen exchange on the triazine ring.

Alkoxy- and aryloxy-s-triazines. Preparation of these compounds is similar to the aminotriazines, but replacement of the second and third chlorine atom of a phenoxy-*s*-triazine with a phenoxy group takes place much more readily and over a much smaller temperature range (3). The first chlorine is substituted at 0° to 5° C., the second at 15° to 20° C., and the third at 30° to 40° C. For each replacement a mole of phenol in aqueous sodium hydroxide was added slowly. With phenol itself, the product obtained by the reaction at 0° to 5° C. had a wide melting range and a low chlorine analysis. This was apparently due to the presence of the diphenoxymonochloro-*s*-triazine which could not be separated by fractional crystallization.

In the preparation of 2,4-dichloro-6-(*p*-chlorophenoxy)-*s*-triazine the

crude product could be separated into two fractions by recrystallization from hexane. The first crystals obtained were 2-chloro-4,6-bis(*p*-chlorophenoxy)-*s*-triazine. After concentrating the solution, crystals of 2,4-dichloro-6-(*p*-chlorophenoxy)-*s*-triazine were obtained. The tendency for the formation of diphenoxy-*s*-triazines at 0° to 5° C., the temperature at which the monosubstituted derivatives are normally formed, was observed only with the more weakly acidic phenols. It has not been determined whether this is due to activation of the remaining chlorine atoms on the triazine ring or to catalysis of the reaction by hydroxyl ions, or a combination of these influences. When acidity of the phenoxy group was higher, as with 2,4-dichlorophenol, clear-cut reactions resulted for replacement of one, two or three chlorine atoms of the cyanuric chloride.

Methoxy derivatives of cyanuric chloride, included for comparative reasons, were prepared in a different way than those for the phenoxy derivatives (2). In the synthesis of 2,4-dichloro-6-methoxy-*s*-triazine, 0.8 mole of sodium bicarbonate and 0.4 mole of cyanuric chloride were added to a mixture of 13 moles of methanol and 3 moles of water. The mixture was stirred at 30° C. until evolution of CO₂ ceased, then diluted with water. The solid which separated was collected on a filter, washed with water, dried and recrystallized. 2-Chloro-4,6-dimethoxy-*s*-triazine was prepared in a similar manner by adding 0.4 mole of sodium bicarbonate and 0.2 mole of cyanuric chloride to a mixture of 3 moles of methanol and 0.5 mole of water. CO₂ evolved at a moderate rate as the temperature rose to 40° C. It was then cooled, diluted with water and filtered. Nineteen aryl-oxo- and alkoxy-*s*-triazines are listed in Table III with their physical properties.

METHODS OF BIOLOGICAL EVALUATION

The fungitoxicity of the compounds described above was determined by subjecting each material to four standard evaluation procedures: spore inhibition, foliage protection, seed protection, and fabric preservation.

The test tube dilution technique (1) was used for determining inhibition of spore germination. Spores from 10-day-old cultures of *Alternaria oleracea* and *Monilinia fructicola* served as test objects. Chemicals were prepared for testing by suspending or dissolving in distilled water at an initial concentration of 1,000 p.p.m. by first adding a solvent, usually acetone, to make a final volume of 5 per cent and an emulsifier (Triton X-155) to equal 0.01 per cent. Test compounds were given alphabetical ratings which corresponded to the concentration that inhibited germination of half of the spores (ED₅₀) in the test drops: AAA=0.01 to 0.1 p.p.m.; AA=0.1 to 1.0 p.p.m.; A=1.0 to 10 p.p.m.; B=10 to 100 p.p.m.; C=100 to 1000 p.p.m.; and D=>1000 p.p.m. (14). Fungicides with known performance for a given use were included in all evaluations for

comparative purposes. In the spore germination on slides, copper sulfate was used as a standard where an A rating for *Alternaria oleracea* and an AA rating for *Monilinia fructicola* were consistently reproducible. Concentrations were based on copper present.

In the foliage disease test (8, 10), which measured the ability of the test chemicals to protect tomato foliage against infection by the early

TABLE III

ARYLOXY- AND ALKOXY-*s*-TRIAZINES SYNTHESIZED FOR EVALUATION AS FUNGICIDES

| Substituents | Crystd. from* | M.p. °C. | Lit. value m.p. °C. | Empirical formula | Analysis, % chlorine | |
|---|------------------|-------------|------------------------|---|----------------------|-------|
| | | | | | Calcd. | Found |
| 2,4-Dichloro-6-(aryloxy or alkoxy)-s-triazines | | | | | | |
| Phenoxy | F | 88-109 | | C ₉ H ₅ ON ₃ Cl ₂ | 29.3 | 20.3 |
| <i>o</i> -Chlorophenoxy | F | 95-97 | | C ₈ H ₄ ON ₃ Cl ₃ | 38.5 | 39.1 |
| <i>p</i> -Chlorophenoxy | F | 105-110 | | C ₈ H ₄ ON ₃ Cl ₃ | 38.5 | 36.2 |
| 2,4-Dichlorophenoxy | F | 122-123 | | C ₈ H ₃ ON ₃ Cl ₄ | 45.6 | 45.4 |
| 2,4,5-Trichlorophenoxy | F | 124-134 | | C ₇ H ₂ ON ₃ Cl ₅ | 51.3 | 50.8 |
| 2,3,4,6-Tetrachlorophenoxy | F | 168-171 | | C ₆ HON ₃ Cl ₆ | 56.0 | 56.3 |
| Pentachlorophenoxy | B | 191-212 | | C ₅ N ₃ Cl ₇ | 59.9 | 59.3 |
| β-Naphthoxy | G | 145-154 | | C ₁₃ H ₇ ON ₃ Cl ₂ | 24.3 | 22.3 |
| Methoxy | E | 89-91 | 88-90 | C ₄ H ₃ ON ₃ Cl ₄ | | |
| 2-Chloro-4,6-bis(aryloxy or alkoxy)-s-triazines | | | | | | |
| Bisphenoxy | F | 119-120 | 121-123 | C ₁₅ H ₁₀ O ₂ N ₃ Cl | | |
| Bis(<i>p</i> -methoxyphenoxy) | B | 122.5-124 | | C ₁₇ H ₁₄ O ₄ N ₃ Cl | 9.9 | 10.6 |
| Bis(<i>p</i> -chlorophenoxy) | F | 140-146 | | C ₁₅ H ₆ O ₂ N ₃ Cl ₃ | 28.9 | 29.3 |
| Bis(2-methoxy-4-acetophenoxy) | B | 196-198 | | C ₂₁ H ₁₈ O ₆ N ₃ Cl | 8.0 | 8.6 |
| Bis(2,4-dichlorophenoxy) | E | 172-173 | | C ₁₆ H ₆ O ₃ N ₃ Cl ₆ | 40.5 | 39.9 |
| Bismethoxy | B | 74-76 | 75-76 | C ₆ H ₆ O ₂ N ₃ Cl | | |
| Tris(aryloxy)-s-triazines | | | | | | |
| Triphenoxy | B | 229-230 | 232-235 | C ₂₁ H ₁₅ O ₃ N ₃ | | |
| Diphenoxy- <i>o</i> -chlorophenoxy | F | 163-165 | | C ₂₁ H ₁₄ O ₃ N ₃ Cl | 9.1 | 8.9 |
| Diphenoxy-2,4-dichlorophenoxy | F | 153-157 | | C ₂₁ H ₁₃ O ₃ N ₃ Cl ₂ | 16.6 | 16.5 |
| Tris(2,4-dichlorophenoxy) | — | 119-121 | | C ₂₁ H ₉ O ₃ N ₃ Cl ₆ | 37.8 | 37.9 |

* Code for solvents used in recrystallization: A=acetone; B=benzene; C=cyclohexanone; D=dioxane; E=benzene-hexane; F=hexane; G=trichloroethylene.

blight fungus, *Alternaria solani*, and the late blight fungus, *Phytophthora infestans*, compounds possessing activity were checked in dosage series at four concentrations adjusted to the protective ability of the test compound. Preparation of chemicals for testing was identical to that described above for the spore inhibition test except the initial concentration was 2000 p.p.m. and the concentration of the solvent was 4.0 per cent. Foliage disease results were converted to percentage disease control based on the number of lesions obtained on the control plants. These data were plotted on logarithmic-probability paper and ED₉₅ values inter-

polated. Standards commonly used included wettable powder formulations of zinc ethylenebis(dithiocarbamate) [zineb], manganous ethylenebis(dithiocarbamate) [maneb], or *N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide [captan]. The average ED₉₅ values on an active ingredient basis against early blight were 40, 30, and 60 p.p.m. respectively and for late blight 25, 20, and 50 p.p.m. respectively.

For evaluation as seed protectants, the chemicals were applied to pea seed (9) in dust form at a dosage of 0.48 per cent by seed weight by placing the chemical and seed in a jar and rotating on a rolling machine for 30 minutes. Lots of 25 seeds per row, 8 rows per flat were sown at random in replication, covered to the same depth and uniformly watered. Untreated seed and seed treated with a standard commercial fungicide, usually bis(dimethylthiocarbamoyl)disulfide [thiram], were included in each test in addition to a control planted in sterilized soil. Flats were stacked at room temperature until the seedlings began to emerge and then removed to the greenhouse. Records were taken at 14 days and converted to percentage stand.

Ability of these chemicals to protect fabric from degradation was determined by the soil burial method. Duplicate strips of 8-ounce cotton duck 1.5 inches by 6 inches with the long dimension parallel to the warp were treated by dipping them for 10 seconds into a 2.0 per cent solution of the test chemical dissolved in acetone or other suitable solvent. They were allowed to dry and then placed vertically in wooden boxes containing soil infested with cellulolytic fungi. Standard reference chemicals and controls were also included in the test box which was incubated at 26° C. for about two weeks. At the end of the exposure period the test specimens were removed from the soil bed and if not completely degraded, gently washed to remove soil, air dried, and tensile strength determined on a Scott tensilometer. Strips were replicated six times in advanced comparative evaluations. Either copper 8-quinolinolate [Quindex] or 2,2'-methylenebis(4-chlorophenol) [G-4] was used as a standard and the treatment procedure was the same as for the test compounds. All concentrations were on an active ingredient basis. Average results for G-4 under the conditions of the test described were 82, 45, and 12 per cent tensile strength retained at immersion concentrations of 1.0, 0.5, and 0.25 per cent chemical after 14 days; for Quindex the values were 94, 60, and 30 per cent tensile strength at 0.5, 0.25, and 0.125 per cent chemical after 14 days.

EXPERIMENTAL RESULTS

The fungitoxicity of five classes of *s*-triazines will be discussed in decreasing order of activity. Several generalizations may be helpful to set the stage for the relationships of structure to activity to be presented. The replacement of more than one chlorine of cyanuric chloride usually yielded

products of inferior fungitoxicity which means that two chlorine atoms must remain on the triazine nucleus for maximum fungicidal activity. None of the compounds evaluated with one chlorine or no chlorine on the triazine ring was among the highly fungitoxic *s*-triazines. As more than one chlorine was added to the benzene ring in both aryloxy- and arylamino-dichloro-*s*-triazine derivatives fungitoxic action decreased. The arylamino-dichloro-*s*-triazines have a broader spectrum of fungicidal activity and are more stable than the corresponding aryloxy compounds.

COMPOUNDS WITH TWO CHLORINES ON THE TRIAZINE RING

2,4-Dichloro-6-(arylamino or alkylamino)-s-triazines. Of all the triazines tested, the most active fungicides were found in the class 2,4-dichloro-6-arylamino-*s*-triazines. Almost half of the triazines synthesized and tested were in this group (Table IV).

The anilino derivative, or parent structure for the arylamino compounds in this class, was highly fungitoxic to germinating spores, had activity in the fabric-preservation test, but was a rather poor foliage protectant. Foliage-protective ability would not be an advantage for this chemical, however, since it is also phytotoxic. The *N*-methylanilino derivative was not phytotoxic, but was not an improved fungicide over the parent structure. The *N*-ethylanilino compound, on the other hand, was a more strikingly effective foliage protectant with some improvement in activity noted in the slide and fabric tests. It was about 90 times more effective against early blight and six times more effective against the late blight organism. This chemical compares favorably as a foliage protectant with the highly active derivatives in this class. The α - and β -naphthylamino isomers were without significant activity as seed protectants or fabric preservatives but were somewhat better than the parent compound as foliage protectants. The α -naphthylamino isomer was several times more effective than the *beta*.

s-Triazines with no fungitoxic action to the most active *s*-triazines evaluated were obtained when substituents were put on the benzene ring. When cyano, nitro, phenylazo, and phenyl groups were put in the *para* position little fungitoxic action was found. However, halogens and/or methyl groups on the benzene ring brought the fungicidal potential of triazine compounds into focus. The position of these groups on the benzene ring is almost as important as the group itself. Arylamino-*s*-triazines with halogens (chlorine or bromine) or methyl groups in the *ortho* position on the benzene ring were better foliage-protectant fungicides than isomers with these substituents in the *para* position. Placement in the *meta* position resulted in chemicals which were least active. A striking example is the comparison of results with the *o*- and *p*-toluidino derivatives. In this case the *ortho* derivative is superior not only as a foliage protectant but

TABLE IV
FUNGICIDAL EFFECTIVENESS OF 2,4-DICHLORO-6-(ARYLAMINO OR ALKYLAMINO)-S-TRIAZINES

| Substituent in the 6 position | Spore inhibition ED ₅₀ range* | | Tomato blight control ED ₉₅ in p.p.m. | | Pea seed protection | | Fabric preservation | | |
|-------------------------------------|---|---|---|---|---|-----------------|---|----|----|
| | <i>Alter- naria oleracea</i> | <i>Moni- linia fructi- cola</i> | <i>Alter- naria solani</i> | <i>Phytoph- thora infestans</i> | Percentage stand after 14 days at 0.48% by seed wt. | Mean control | Percentage tensile strength retained after 14 days at 2.0% 1.0% 0.5% | | |
| Anilino | AAA | B | 8,000 | P** | 37 | I | 101 | 78 | 0 |
| N-Methylanilino | A | B | 9,000 | I,200 | 16 | 14 | 90 | 0 | 0 |
| N-Ethylanilino | ≧AA | B | 110 | 200 | 4 | 8 | 59 | 47 | 7 |
| o-Toluidino | AA | A | 80 | 120 | 50 | 5 | 81 | 92 | 47 |
| p-Toluidino | ≧AA | A | 5,000 | 1,000 | 40 | 14 | 90 | 0 | 0 |
| o-Ethylanilino | AA | A | 90 | 120 | 30 | 5 | 88 | 85 | 0 |
| o-Methoxyanilino | ≧AA | C | 250 | 500 | 54 | 37 | 0 | 0 | 0 |
| p-Methoxyanilino | AA | B | 250 | 150 | 16 | 5 | 28 | 0 | 0 |
| o-Chloroanilino | AAA | A | 80 | 80 | 54 | I | 102 | 86 | 48 |
| m-Chloroanilino | AA | A | 750 | 350 | 26 | 5 | 106 | 0 | 0 |
| p-Chloroanilino | AAA | A | 300 | 200 | 69 | 5 | 85 | 76 | 69 |
| 2,4-Dichloroanilino | AAA | A | 200 | 250 | 38 | 8 | 68 | 0 | 0 |
| 2,5-Dichloroanilino | AA | A | 2,500 | 7,000 | 30 | 14 | 0 | 0 | 0 |
| 3-Chloro-2-methylanilino | ≧AA | A | 300 | 400 | 14 | 8 | 12 | 0 | 0 |
| 5-Chloro-2-methylanilino | ≧AA | A | 115 | 1,300 | 14 | 18 | 74 | 0 | 0 |
| o-Bromoanilino | AAA | A | 60 | 100 | 37 | 16 | 49 | 0 | 0 |
| m-Bromoanilino | AAA | A | 275 | 150 | 26 | 8 | 41 | 0 | 0 |
| p-Bromoanilino | AAA | A | 250 | 90 | 58 | 19 | 95 | 61 | 0 |
| p-Cyanoanilino | A | D | >10,000 | >10,000 | 0 | I | 0 | 0 | 0 |
| p-Nitroanilino | AAA | D | >10,000 | >10,000 | 32 | I | 0 | 0 | 0 |
| p-Phenylazoanilino | D | D | >10,000 | >10,000 | 0 | I | 0 | 0 | 0 |
| α-Naphthylamino | AA | A | 1,100 | 1,100 | 5 | I | 0 | 0 | 0 |
| β-Naphthylamino | AA | C | 2,500 | 9,000 | 22 | 30 | 0 | 0 | 0 |
| o-Phenylanilino | A | D | 4,000 | 7,000 | 0 | I | 0 | 0 | 0 |
| p-Phenylanilino | C | D | >10,000 | >10,000 | 0 | I | 0 | 0 | 0 |
| Diisopropylamino | C | D | >10,000 | >10,000 | 6 | 18 | 0 | 0 | 0 |
| n-Butylamino | ≧AA | A | P | P | 8 | 18 | 112 | 98 | 2 |

* AAA=0.01 to 0.1 p.p.m.; AA=0.1 to 1.0 p.p.m.; A=1 to 10 p.p.m.; B=10 to 100 p.p.m.; C=100 to 1,000 p.p.m.; D=>1,000 p.p.m.; ≧ indicates toxicity equal to or greater than.

** P indicates phytotoxic to test plant at 0.2 per cent.

also as a seed protectant and fabric preservative. Interestingly enough this difference does not appear in the spore germination evaluation. The relative foliage-protectant activity of the *o*- and *p*-phenylanilino isomers is another example of the superiority of the former isomer although the level of activity is low. An exception to this effect, however, was demonstrated in the case of the *o*- and *p*-methoxyanilino isomers. The magnitude of these differences and relationships can be clearly seen by examination of Table IV.

Monochloro and monobromo substitution on the benzene ring resulted in triazines with highest fungicidal efficiency. No differences were noted in the slide germination ratings that could be ascribed to positional effects of bromine on the benzene ring, but the *m*-chloro isomer was significantly less active against *Alternaria oleracea* than the *ortho* or *para*

isomers which were about equally active. Substitution of chlorine or bromine in the *meta*, *para*, and *ortho* positions on the benzene ring improved foliage-protective ability in the order stated. The chloro and bromo derivatives were about equally effective for this application. The *para* isomers, both chloro and bromo, were more effective as seed protectants and fabric preservatives and the chloro compound was significantly more active than the bromo. Not only did the chlorine derivatives have a broader spectrum of fungicidal activity but from a practical point of view they are more inviting as potential fungicides because of cost. 2,4-Dichloro-6-(*o*-chloroanilino)-*s*-triazine is considered the most efficient over-all triazine fungicide; however, the *p*-chloro isomer and the *o*-toluidino derivative follow very closely. When these chemicals were ground in good wettable powder formulations their foliage-protectant ability compared very favorably with commercially successful fungicides. The ED₉₅ values for protection against early and late blight for 2,4-dichloro-6-(*o*-chloroanilino and *o*-toluidino)-*s*-triazine were 25, 50 and 65, 80 respectively. Information on improvement of foliage-protectant ability and effect on phytotoxicity by formulation will follow in another paper.

Fungicidal efficiency was reduced when two chlorines were on the benzene ring. Although the 2,4-dichloroanilino derivative was an effective foliage protectant, it was inferior to the *o*-chloro compound. When chlorines were in the 2,5-positions, a sharp loss in foliage-protective ability was detected. The dichloro compounds were also less effective in the soil tests. A combination of chloro and methyl groups on the benzene ring resulted in a similar effect.

Information is given on only two alkylamino-*s*-triazines. The diisopropylamino compound was without significant fungicidal properties but the *n*-butylamino derivative was very fungitoxic to spores and among the most active fabric preservatives. It also had the distinction of being the most phytotoxic of all triazines tested which excluded it as a foliage protectant candidate (Table IV).

Halogenated 2,4-dichloro-6-arylamino-s-triazines. As discussed in a previous section of this paper the high fungitoxicity and particularly the excellent foliage-protectant ability of the monochloro and bromo compounds in this class led to the preparation of a series of reaction products by direct halogenation of the benzene ring of 2,4-dichloro-6-anilino-*s*-triazine and 2,4-dichloro-6-(*o*-toluidino)-*s*-triazine. The over-all result of this effort can be summarized very briefly by saying none of the reaction products formed were found to be better fungicides than the 2,4-dichloro-6-(*o*-chloroanilino or *o*-toluidino)-*s*-triazines (Table V).

There are, however, several interesting side lights on these materials. Analysis showed that the chlorinated anilino material (40.6 per cent Cl) was essentially a mixture of the 2,4-dichloro-6-(*o*- and *p*-chloroanilino)-*s*-

TABLE V

FUNGICIDAL EFFECTIVENESS OF HALOGENATED 2,4-DICHLORO-6-(ARYLAMINO)-5-TRIAZINES

| Substituent in the 6 position | Spore inhibition ED ₅₀ range* | | Tomato blight control ED ₉₅ in p.p.m. | | Pea seed protection | | Fabric preservation | | |
|-------------------------------------|---|---------------------------------|---|-----------------------------------|---|-----------------|---|----|---|
| | <i>Alternaria oleracea</i> | <i>Monilinia fructicola</i> | <i>Alternaria solani</i> | <i>Phytophthora infestans</i> | Percentage stand after 14 days at 0.48% by seed wt. | Mean control | Percentage tensile strength retained after 14 days at 2.0% 1.0% 0.5% | | |
| Chlorinated anilino (40.6% Cl) | ≥ AAA | A | 160 | 120 | 50 | 6 | 101 | 74 | 1 |
| Dichlorinated anilino (45.8% Cl) | ≥ AAA | A | 190 | 500 | 56 | 6 | 49 | 0 | 0 |
| Chlorinated- <i>o</i> -toluidino | ≥ AA | A | 130 | 170 | 44 | 14 | 26 | 0 | 0 |
| Brominated anilino (34.6% Br) | ≥ AAA | A | 250 | 350 | 46 | 6 | 87 | 0 | 0 |
| Brominated anilino (39.9% Br) | ≥ AAA | A | 200 | 90 | 54 | 6 | 84 | 0 | 0 |
| Brominated- <i>o</i> -toluidino | ≥ AA | A | 130 | 170 | 44 | 14 | 0 | 0 | 0 |
| Brominated <i>N</i> -methylanilino | ≥ AA | ≥ AA | 250 | 300 | 16 | 8 | 86 | 0 | 0 |
| Brominated <i>N</i> -ethylanilino | ≥ AA | ≥ AA | 150 | 200 | 20 | 8 | 51 | 0 | 0 |

* AAA=0.01 to 0.1 p.p.m.; AA=0.1 to 1.0 p.p.m.; A=1 to 10 p.p.m.; B=10 to 100 p.p.m.; C=100 to 1,000 p.p.m.; D=>1,000 p.p.m.; ≥ indicates toxicity equal to or greater than.

triazine isomers with the latter isomer predominating by a ratio of about 3:1. On this basis it would be predicted that this reaction product would be between the two pure isomers in fungicidal effectiveness and the results show this to be true for foliage-protective ability.

The reaction products of the chlorination of the anilino- and *o*-toluidino-*s*-triazines were the most promising of all the materials in the directly halogenated series. The interest in the latter material was as a foliage-protectant fungicide. Analysis showed this product was essentially a monochloro derivative making it comparable to either 2,4-dichloro-6-(3-chloro-2-toluidino or 5-chloro-2-toluidino)-*s*-triazine. These two chemicals were discussed in the previous section and it would appear that the reaction product was somewhat more effective as a foliage protectant than either of the pure chemicals. An explanation for this effect is not obvious.

2,4-Dichloro-6-(aryloxy or alkoxy)-*s*-triazines. Although some of the 2,4-dichloro-6-aryloxy-*s*-triazines were found to be very active fungicides, none of the nine chemicals in this class were equal in over-all fungitoxicity to their arylamino analogues. Furthermore these chemicals were more phytotoxic and less stable than the amino-*s*-triazines. None of these chemicals protected fabric in the soil burial test (Table VI).

Monochloro substitution on the benzene ring of the phenoxy parent compound, as in the anilino-*s*-triazine analogue, sharply increased ability to protect foliage. In this case the *para* isomer was more fungitoxic than the *ortho* compound as measured by both the foliage-protectant

TABLE VI
FUNGICIDAL EFFECTIVENESS OF 2,4-DICHLORO-6-(ARYLOXY OR ALKOXY)-*s*-TRIAZINES

| Substituent in the 6 position | Spore inhibition ED ₅₀ range* | | Tomato blight control ED ₉₅ in p.p.m. | | Pea seed protection | | Fabric preservation |
|-------------------------------------|---|---|---|---|---|-----------------|---|
| | <i>Alter- naria oleracea</i> | <i>Moni- linia fructi- cola</i> | <i>Alter- naria solani</i> | <i>Phytoph- thora infestans</i> | Percentage stand after 14 days at 0.48% by seed wt. | Mean control | Percentage tensile strength retained after 14 days at 2.0% |
| Phenoxy | A | A | 5,000 | >10,000 | 8 | 8 | 0 |
| <i>o</i> -Chlorophenoxy | A | A | 350 | 350 | 46 | 16 | 0 |
| <i>p</i> -Chlorophenoxy | ≥AA | ≥AA | 170 | 300 | 10 | 16 | 0 |
| 2,4-Dichlorophenoxy | A | A | 350 | 650 | 60 | 1 | 0 |
| 2,4,5-Trichlorophenoxy | A | A | 1,500 | 1,700 | 14 | 1 | 0 |
| 2,3,4,6-Tetrachlorophenoxy | A | A | 1,700 | 2,000 | 4 | 16 | 0 |
| Pentachlorophenoxy | D | D | P** | P | 0 | 14 | 48 |
| β-Naphthoxy | AA | AA | 1,600 | 1,000 | 28 | 1 | 0 |
| Methoxy | ≥AA | ≥AA | >10,000 | >10,000 | 18 | 18 | 0 |

* AAA=0.01 to 0.1 p.p.m.; AA=0.1 to 1.0 p.p.m.; A=1 to 10 p.p.m.; B=10 to 100 p.p.m.; C=100 to 1,000 p.p.m.; D=>1,000 p.p.m.; ≥ indicates toxicity equal to or greater than.

** P indicates phytotoxic to test plant at 0.2 per cent.

and spore-germination tests. The 2,4-dichloro member of this class was one of the more efficient triazines for the protection of seed although the level of activity was low compared to commercially used chemicals. Foliage-protectant ability decreased from the monochloro to the pentachlorophenoxy derivative and phytotoxicity increased. It seems worthwhile to point out again that when the number of chlorine atoms on the benzene ring was greater than one on both the 2,4-dichloro-6-(arylamino and aryloxy)-*s*-triazines foliage-protective ability decreased. The methoxy analogue in this class was very fungitoxic to spores but worthless when applied as a foliage protectant.

COMPOUNDS WITH ONE CHLORINE ON THE TRIAZINE RING

2-Chloro-4,6-bis(arylamino or alkylamino)-s-triazines. Of the eight chemicals tested in this class only the bis(2,5-dichloroanilino) derivative could be classed as having significant fungicidal properties (Table VII). Contrary to previous structure to activity relationships, the bis(2,5-dichloroanilino) compound was more fungitoxic than the bis(monochloroanilino) derivative. However, as in the triazine classes previously discussed, a single chlorine atom on the benzene ring increased activity.

2-Chloro-4,6-bis(aryloxy or alkoxy)-s-triazines. The fungicidal stature of this class of *s*-triazines was lower than the bis(amino) derivatives (Table VIII). Placement of substituents on the benzene ring did not increase activity. The moderate fabric-preservative activity of the methoxy analogue is rather puzzling since none of the other compounds with one chlorine on the triazine ring has displayed ability to protect fabric. In a

TABLE VII

FUNGICIDAL EFFECTIVENESS OF 2-CHLORO-4,6-BIS(ARYLAMINO OR ALKYLAMINO)-S-TRIAZINES

| Substituents in the 4,6 positions | Spore inhibition ED ₅₀ range* | | Tomato blight control ED ₉₅ in p.p.m. | | Pea seed protection | | Fabric preservation |
|---|---|---|---|---|---|-----------------|---|
| | <i>Alter- naria oleracea</i> | <i>Moni- linia fructi- cola</i> | <i>Alter- naria solani</i> | <i>Phytoph- thora infestans</i> | Percentage stand after 14 days at 0.48% by seed wt. | Mean control | Percentage tensile strength retained after 14 days at 2.0% |
| Bisanilino | C | C | > 10,000 | > 10,000 | 22 | 14 | 0 |
| Bis(<i>N</i> -methylanilino) | C | D | 6,000 | 6,000 | 12 | 14 | 0 |
| Bis(<i>p</i> -chloroanilino) | B | B | > 10,000 | 8,000 | 20 | 14 | 0 |
| Bis(2,5-dichloroanilino) | AA | AA | 1,500 | 1,000 | 7 | 1 | 0 |
| Bis(<i>p</i> -methoxyanilino) | C | C | > 10,000 | > 10,000 | 8 | 14 | 0 |
| Bis(<i>p</i> -nitroanilino) | ≥ AA | C | 6,000 | 2,500 | 2 | 1 | 0 |
| Bis(α-naphthylamino) | D | D | > 10,000 | > 10,000 | 6 | 14 | 0 |
| Bis(<i>n</i> -butylamino) | D | D | > 10,000 | > 10,000 | 14 | 18 | 0 |

* AAA=0.01 to 0.1 p.p.m.; AA=0.1 to 1.0 p.p.m.; A=1 to 10 p.p.m.; B=10 to 100 p.p.m.; C=100 to 1,000 p.p.m.; D=>1,000 p.p.m.; ≥ indicates toxicity equal to or greater than.

TABLE VIII

FUNGICIDAL EFFECTIVENESS OF 2-CHLORO-4,6-BIS(ARYLOXY OR ALKOXY)-S-TRIAZINES

| Substituents in the 4,6 positions | Spore inhibition ED ₅₀ range* | | Tomato blight control ED ₉₅ in p.p.m. | | Pea seed protection | | Fabric preservation |
|---|---|---|---|---|---|-----------------|---|
| | <i>Alter- naria oleracea</i> | <i>Moni- linia fructi- cola</i> | <i>Alter- naria solani</i> | <i>Phytoph- thora infestans</i> | Percentage stand after 14 days at 0.48% by seed wt. | Mean control | Percentage tensile strength retained after 14 days at 2.0% |
| Bisphenoxy | A | D | 1,500 | 1,500 | 16 | 15 | 0 |
| Bis(<i>p</i> -methoxyphenoxy) | D | D | > 10,000 | > 10,000 | 32 | 18 | 0 |
| Bis(<i>p</i> -chlorophenoxy) | B | B | 300 | 7,000 | 30 | 15 | 0 |
| Bis(2-methoxy-4-acetophenoxy) | D | C | 4,000 | 3,000 | 6 | 15 | 0 |
| Bis(2,4-dichlorophenoxy) | C | C | > 10,000 | > 10,000 | 10 | 14 | 0 |
| Bismethoxy | B | B | > 10,000 | > 10,000 | 14 | 18 | 81 |

* AAA=0.01 to 0.1 p.p.m.; AA=0.1 to 1.0 p.p.m.; A=1 to 10 p.p.m.; B=10 to 100 p.p.m.; C=100 to 1,000 p.p.m.; D=>1,000 p.p.m.; ≥ indicates toxicity equal to or greater than.

follow-up test at a 1.0 per cent concentration no activity was detected. It is clear from these results and also the results discussed previously on the 2-chloro-4,6-bis(arylamino and alkylamino)-s-triazine compounds that replacement of the second chlorine of cyanuric chloride sharply reduced fungitoxicity.

COMPOUNDS WITH NO CHLORINE ON THE TRIAZINE RING

Tris(aryloxy)-s-triazines. Only four chemicals were prepared in this class since fungitoxicity appeared to be lost when the third chlorine on cyanuric chloride was replaced (Table IX).

TABLE IX
FUNGICIDAL EFFECTIVENESS OF TRIS(ARYLOXY)-*s*-TRIAZINES

| Aryloxy substituents | Spore inhibition ED ₅₀ range* | | Tomato blight control ED ₉₅ in p.p.m. | | Pea seed protection | | Fabric preservation |
|------------------------------------|---|-------------------------------|---|--------------------------------|---|--------------|--|
| | <i>Alter-naria oleracea</i> | <i>Moni-linia fructi-cola</i> | <i>Alter-naria solani</i> | <i>Phytoph-thora infestans</i> | Percentage stand after 14 days at 0.48% by seed wt. | Mean control | Percentage tensile strength retained after 14 days at 2.0% |
| Triphenoxy | C | D | > 10,000 | > 10,000 | 44 | 15 | 0 |
| Diphenoxy- <i>o</i> -chlorophenoxy | D | D | > 10,000 | > 10,000 | 14 | 15 | 0 |
| Diphenoxy-2,4-dichlorophenoxy | D | C | 8,000 | 6,000 | 18 | 15 | — |
| Tris(2,4-dichlorophenoxy) | C | D | > 10,000 | > 10,000 | 6 | 14 | 0 |

* AAA=0.01 to 0.1 p.p.m.; AA=0.1 to 1.0 p.p.m.; A=1 to 10 p.p.m.; B=10 to 100 p.p.m.; C=100 to 1,000 p.p.m.; D=>1,000 p.p.m.; ≥ indicates toxicity equal to or greater than.

DISCUSSION

Placement of a halogen or methyl group on the benzene ring of 2,4-dichloro-6-(anilino)-*s*-triazine, as measured by a series of bioassays, resulted in several highly effective triazine fungicides. These chemicals are among the more efficient organic compounds now known for ability to inhibit germination of fungus spores, control foliage diseases, and prevent fabric deterioration. Why this particular configuration of the triazine molecule possesses maximum fungicidal efficiency is not evident from the present data. Such knowledge would contribute to the understanding of the mechanism of fungicidal action.

The parent starting material, cyanuric chloride, hydrolyzes very easily and rapidly when exposed to water and possesses no fungicidal properties. It has been shown that when all the chlorines of cyanuric chloride were replaced no fungicidal tendencies were detected; when one chlorine was present on the triazine ring significant fungicidal activity could be measured; when two chlorines were present the stage was set for development of maximum fungicidal effectiveness. From a chemical point of view these two chlorines are labile and would be considered as very reactive. It would seem only logical that they would be able to react with biological systems, and it is clear they play a vital function in the fungitoxic action of triazines.

Most authorities agree that the properties of an effective fungicide must be such that they are able to penetrate the cell wall and membranes of the spore efficiently and thereby gain access to the site of vital cell functions. This would pose the question whether the replacement of the chlorine on the triazine ring reduces the penetrability of the chemical or its ability to interfere with vital cell function. One could speculate that as the chlorine on the triazine ring is replaced with larger groups the physi-

cal dimensions of the molecule are increased and therefore it is more difficult for the molecule to pass through the spore wall and membranes on the basis of sheer size. This does not seem likely, however, since even larger molecules are known to be good fungicides. If size were an important factor it would be expected, for example, that 2-chloro-4,6-bis(methoxy)-*s*-triazine would be significantly more fungitoxic than any of the compounds in the 2-chloro-4,6-bis(aryloxy or alkoxy)-*s*-triazine class (Table VIII) but this is not the case.

When substituents are placed on the benzene ring of 6-(aryloxy or arylamino)-2,4-dichloro-*s*-triazines, fungitoxicity may be increased or decreased. Methyl groups, bromine, and chlorine increased activity while nitro, cyano, phenyl and phenylazo groups decreased activity. Not only is the type of substituent important but also its position on the ring. It seems reasonable to assume that any of these changes probably increase or decrease, as the case may be, the reactivity of the chlorines on the triazine ring in addition to altering the partition coefficient by changing lipid and water solubilities. A position change on the molecule may not influence just one effect but may promote a series of interactions. It seems sensible that these changes be looked at as a function of the molecule as a whole. One could list a large number of examples where a particular group or position of a group had a certain effect on fungitoxicity, but such an approach would not seem feasible because such comparisons, even on the same basic nucleus, may have very different effects. For example, in the case of the 2,4-dichloro-6-arylamino-*s*-triazines foliage-protective ability was best when chlorine was placed in the *ortho* position on the benzene ring while in the case of the aryloxy derivatives it was necessary to have the chlorine in the *para* position for best foliage protection. Since only fungitoxicity is measured in most studies of this kind, interpretation must be confined to these terms.

On the basis of the laboratory information presented and field information to be included in another paper, 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine, coded B-622, is considered as having a good chance of becoming a commercially successful fungicide.

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CHEMICAL STRUCTURES AND DISSOCIATION CONSTANTS
OF AMINO ACIDS, PEPTIDES, AND PROTEINS IN
RELATION TO THEIR REACTION RATES WITH
2,4-DICHLORO-6-(*o*-CHLOROANILINO)-
s-TRIAZINE

H. P. BURCHFIELD AND ELEANOR E. STORRS

SUMMARY

The susceptibility to alkylation of sixty-one amino acids, peptides, co-enzymes, macromolecules, and related compounds by 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine has been investigated. Aspartic acid is the least reactive compound on which quantitative data were obtained with a second order velocity constant of 1.2×10^{-3} . With the exceptions of tyrosine and cysteine, the commonly occurring amino acids react from 2 to 10 times more rapidly than aspartic acid. Formation of peptide chains has been shown to increase the reactivity of the terminal amino groups in three series of compounds terminating in glycine, α -alanine, and leucine residues. The materials most susceptible to alkylation are glutathione, cysteine, *p*-aminobenzoic acid, hydroxyproline, pyridoxamine, proline, tyrosine, and pyridoxine.

Many anomalies in order of reactivity can be explained if it is assumed that only the $R-NH_2$ and $R-S^-$ groups can take part in the reactions with the $R-NH_3^+$ and $R-SH$ groups excluded. When velocity constants are recalculated taking into account the dissociation constants of the compounds a different order of reactivity is obtained. Thus peptides are intrinsically less, rather than more, reactive than amino acids, while proline is more reactive than hydroxyproline. *p*-Aminobenzoic acid which apparently is one of the most reactive compounds becomes one of the least reactive. It has been shown that nucleophilicity can be correlated with basicity for a group of amino acids with various substituents on the α -carbon atom.

The modified second order rate equation contains a term for hydrogen-ion concentration which indicates that observed reaction rates should decrease 10-fold for each unit decrease in pH in the physiological range. This was observed to be the case for cysteine and glycine from pH 7.0 to pH 5.0. It is possible that compounds whose biological activity depends upon their ability to alkylate essential metabolites would be less effective against organisms with lower internal pH values.

The susceptibility of proteins to alkylation also varies considerably. Thus edestin does not combine with the triazine under the conditions employed, while reaction with egg albumin takes place at a slow but measurable rate. Since reactivity increases with degree of dissociation it is suggested that the reactivities of protein molecules could be potentiated if their functional groups were located in environments which would permit a greater degree of dissociation than would occur with a free amino acid at the same pH.

INTRODUCTION

The biological activity of the *s*-triazines (5, 31, 36), as well as that of certain other fungicides (10, 18), herbicides (11), and nematocides (26), probably depends upon the presence of an active halogen atom in the

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molecule which is capable of undergoing displacement reactions with compounds essential to metabolism, thus removing them from the sphere of action. In the case of 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine, the halogen atoms attached to the heterocyclic ring are reactive since they are adjacent to C=N bonds, while the one attached to the benzene ring is inert although it exerts modifying effects on the physico-chemical properties of the molecule (4, 5).

The *s*-triazines are thus capable of reacting with constituents of protoplasm containing amino and sulfhydryl groups such as amino acids, peptides, co-factors, and proteins, so that spores may fail to germinate and mycelium to grow through multiple rather than single causes. Any selectivity with regard to sites of action probably depends upon relative reaction rates rather than on absolute specificity for a single receptor site.

In order to determine which compounds within living organisms are likely to be depleted by treatment with the *s*-triazines, the reaction rates of a selected number of metabolic intermediates and macromolecules with 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine were investigated. This paper describes the experimental results and attempts to correlate reactivity with the chemical structures and dissociation constants of the metabolites. A subsequent paper will show how changes in the structures of the *s*-triazines modify their reactivities with typical amino acids.

MATERIALS AND METHODS

Chemicals. Acetyl-DL-alanine, adenine, adenosine triphosphate, DL- α -alanine, β -alanine, DL-alanyl-DL-alanine, DL-alanylglycylglycine, DL-alanyl-DL-phenylalanine, *p*-aminobenzoic acid, L-arginine, L-asparagine, DL-aspartic acid, coenzyme I, cytosine, L-glutamic acid, L-glutamine, glutathione, glycyl-DL-alanine, glycylglycine, glycylglycylglycine, glycyl-DL-leucine, L-histidine, L-hydroxyproline, DL-isoleucine, L-leucine, DL-leucylglycine, DL-leucylglycylglycine, L-lysine, DL-methionine, DL-phenylalanine, L-proline, pyridoxamine, pyridoxine, DL-serine, taurine, thiamine, DL-threonine, thymine, DL-tryptophan, L-tyrosine, and DL-valine were obtained from the Nutritional Biochemicals Corp. Pyridine was obtained from the Mallinckrodt Chemical Works, glycolic acid from the Matheson Co., acetamide, cysteine, indole-3-acetic acid, *p*-hydroxybenzoic acid, *p*-hydroxyphenoxyacetic acid, and nicotinamide from Eastman Kodak Co., and glycine from the Calco Chemical Co. Amylopsin ($2\times$ U.S.P. Pancreatin), egg albumin (crystalline), edestin ($2\times$ cryst.), malt diastase (analytical), pepsin ($2\times$ crystallized), lysozyme (crystalline), trypsinogen ($1\times$ crystalline), urease, ribosenucleic acid (RNA) and desoxyribosenucleic acid (DNA) were obtained from Nutritional Biochemicals Corp. Bovine intestinal phosphatase was obtained from Pentex Inc. The preparation and properties of 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine have been described elsewhere (6, 31).

Determination of solubility. One ml. of an acetone solution of the s-triazine containing 10^4 μ g. per ml. was pipetted into 99 ml. of *M/30* phosphate buffer adjusted to pH 7.0, with swirling. After three hours the suspension was filtered through No. 5 Whatman filter paper and the filtrate centrifuged for 15 minutes at 3,400 r.p.m. A 5-ml. aliquot of the supernatant was then withdrawn for analysis (6).

Measurement of reaction rates. All reaction rate measurements were made at a concentration of 5 μ g. per ml. of triazine in approximately *M/30* aqueous phosphate buffer containing 1 per cent acetone. From 10 to 10^5 mols of substrate per mol of triazine were used depending upon reactivity and pH. The pH was maintained at 7.0 except in cases where it was desired to study reactivity as a function of hydrogen-ion concentration. When large amounts of substrate were used the pH tended to shift somewhat, so in these cases the solutions were titrated back to pH 7.0 with the acid or alkaline component of the buffer. The temperature was maintained at 29° C. in all cases except for studies on the variation of reactivity with pH where it was held at 30° C. De-ionized water was used in making up the buffers, and in the case of compounds containing sulfhydryl groups, the reactions were carried out under an atmosphere of nitrogen to prevent oxidation.

Two methods were used for the determination of reaction rates. In *Method A*, 99 ml. of the buffered substrate were preconditioned at 29° C. and 1 ml. of an acetone stock solution of the triazine added with swirling. A 5-ml. sample was withdrawn for analysis immediately, and additional samples were taken at various time intervals depending upon the reaction rate of the compound under investigation. From 5 to 12 points were obtained for each curve. The reaction was stopped by adding 1 ml. of pyridine-glycine reagent to each sample as described in the analytical procedure (6). The logarithm of the absorbance was plotted against time (Fig. 1), and the absorbance at zero time determined by extrapolation. The half time for the reaction (the time required for the absorbance to reach one-half its initial value) was then interpolated. Under the conditions of the test residual colors with absorbances of 0.08 were still obtained after 24 hours even when the substrate was highly reactive, so the half-time values were corrected for this background effect. Second order reaction rate constants were then calculated using the equation

$$k = \frac{2.303}{t(a-b)} \log \frac{b(a-x)}{a(b-x)} \quad (1)$$

where k is the rate constant, t the time in seconds, b the molar concentration of triazine, a the molar concentration of substrate, and x the amount of triazine which is unreacted at time t .

Method B was carried out in the same manner as *Method A*, except that

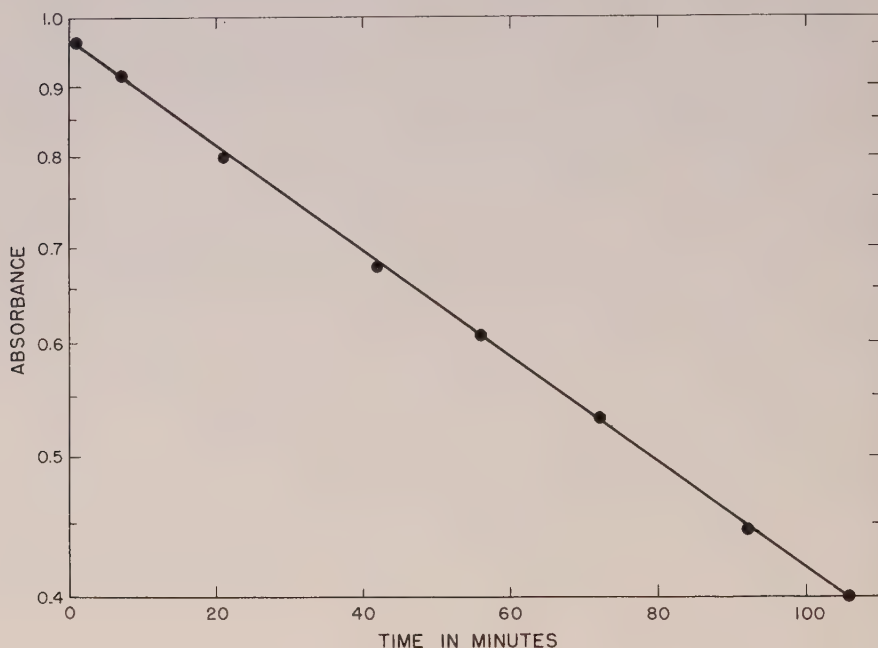


FIGURE 1. Reaction rate of 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine with 10^3 molar equivalents of glycine as determined by decrease in absorbance.

the reaction was stopped by extracting a 10-ml. aliquot with 10 ml. of petroleum ether (b.p. 30 to 60° C.) in a separatory funnel. A 5-ml. aliquot of the ether extract was then evaporated to dryness, and the residue analyzed for triazine by the pyridine-glycine method described previously. When this technique was used it was unnecessary to correct the $t_{1/2}$ values for background absorption since the interfering substance was removed during the extraction. In some of the experiments with proteins emulsions were formed on extraction. It was necessary to break them by centrifugation.

Determination of pK'_b . Values for pK'_b obtained from the literature were taken from the compilation given by Cohn and Edsall (8, p. 85), except for threonine (33). Values for alanylphenylalanine, leucylglycine, and leucylglycylglycine could not be located. These were determined at 24° C. by the method of Kirk and Schmidt (16) using a Beckman Model G pH meter.

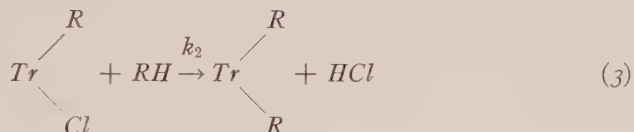
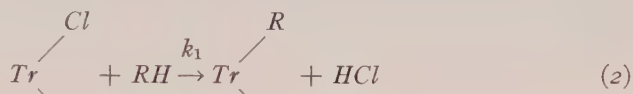
EXPERIMENTAL RESULTS AND DISCUSSION

DEVELOPMENT OF METHOD

The method used to measure the reaction rates of 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine with various metabolites is based on a colori-

metric procedure for the analysis of the *s*-triazines which is dependent upon the presence of an active halogen atom in the molecule (6). When the *s*-triazine reacts with a substrate such as cysteine or glycine, active halogens are replaced and the compound can no longer be detected analytically. This makes it possible to measure reaction rates by determining the decrease in color intensity as a function of time.

The *s*-triazine used in these investigations has two halogen atoms either or both of which can be displaced in reactions with nucleophilic compounds. Thus if the symbol *Tr* is used to designate the *s*-triazine radical, and *RH* some metabolite, the following reactions can take place stepwise.



The original triazine gives a very intense color in the pyridine-alkali reaction (6), materials analogous to the product of equation (2) give moderately intense colors, while materials similar to the product of equation (3) do not react. On this basis it might be assumed that the color measured in the analytical test is the sum of the contributions from the residual *s*-triazine and the product of (2).

When the substrate is present in large excess and color intensity is plotted against time of reaction on semi-logarithmic coordinates, a linear relationship indicative of a pseudounimolecular reaction is invariably obtained (Fig. 1). If consecutive reactions were involved in which $k_1 > k_2$ (which seems likely in view of the conditions required to prepare these compounds) the relationship would be nonlinear if the product of equation (2) made a large contribution to the total color.

In experiments made with cysteine and glycine it was found that faint color with absorbances of about 0.08 were still developed after 24 hours reaction time. Calculations based on the initial stages of the rate curves (Fig. 1) show that less than 0.1 per cent of the original triazine should be unreacted at 24 hours. This should not be detectable. Thus the residual colors appear to be caused by the products of reaction (2) which are relatively stable under these conditions. In view of this, the observed values for half times were corrected for the residual color to a first approximation. This correction together with the fact that the curves are linear to within error of measurement insures that the second order rate constant for equation (2) is the quantity which is being measured.

The reaction between the *s*-triazine and substrate is stopped by adding a large excess of pyridine-glycine reagent to the reaction mixture. This is capable of reacting with 50 per cent of the remaining triazine within 0.03 minute so the error in time due to continuing reaction with the substrate is very small.

The reactions were carried out in homogeneous solution since the original concentration of triazine was maintained at 5.0 $\mu\text{g.}$ per ml. while the solubility of this compound in aqueous buffer containing 1 per cent acetone is 10 $\mu\text{g.}$ per ml.

Errors due to hydrolysis are negligibly small since the half time of the compound in aqueous phosphate buffer is about 3×10^4 minutes at pH 7.0 and 29° C. The concentration of substrate was usually adjusted so that half-time values would be between 20 and 300 minutes.

This procedure (*Method A*) was used successfully for the evaluation of a large number of compounds until the reactivity of glutathione was measured. At first it appeared that this compound might react many thousands of times faster than any other material investigated since two molecular equivalents suppressed almost all of the color due to unreacted triazine in less than one minute. Further investigation showed that the color was suppressed even when the glutathione was added to the pyridine reagent in advance of the triazine, so it was suspected that some effect other than displacement of the active halogens of the triazine might be involved. In order to confirm this, a modified method was developed (*Method B*) in which the reaction was stopped by shaking an aliquot of the reaction mixture with petroleum ether in the expectation that any unreacted triazine would appear in the hydrocarbon phase while the glutathione and buffer would remain in the aqueous phase. When this was done it was found that most of the triazine could be recovered after a 20-minute incubation period with two molecular equivalents of glutathione. From this it was evident that the glutathione was merely serving to repress color development in the earlier experiments and was not combining chemically with the triazine. Reaction rate measurements carried out by this modified method showed it to be about as reactive as cysteine.

Since a large number of measurements had already been made using *Method A*, it became necessary to compare *A* and *B* to determine if the results agreed. The average value for the half time of the *s*-triazine in the presence of 1,000 molecular equivalents of glycine was found to be 75 minutes by *Method A* and 72 minutes by *Method B* (Table I). Evidently the reaction rate is somewhat faster as measured by *Method B* but the correspondence is satisfactory for the purposes of this paper. *Method B* has a distinct advantage in that the reaction products of equation (2) are not extracted into the petroleum ether phase since they generally contain carboxyl or other polar groups. This makes it unnecessary to correct the

results for background absorption and confirms that both *A* and *B* measure the velocity constant of reaction (2).

The use of *Method A* was continued when applicable, since the technique is simpler and the precision of measurement considerably better (Table I). However, a number of other cases ultimately arose in which it could not be used. Thiamine gave some color even in the absence of the triazine and so had to be separated by extraction, while egg albumin, *p*-hydroxybenzoic acid, and *p*-hydroxyphenoxyacetic acid suppressed color

TABLE I
COMPARISON OF METHODS A AND B FOR DETERMINING THE REACTION RATE
OF 2,4-DICHLORO-6-(*p*-CHLOROANILINO)-*s*-TRIAZINE WITH GLYCINE

| Test No. | Half time in minutes | |
|----------|----------------------|----------|
| | Method A | Method B |
| 1 | 74 | 71 |
| 2 | 75 | 69 |
| 3 | 75 | 76 |
| 4 | 75 | 71 |
| 5 | 74 | 69 |
| 6 | 75 | 73 |
| Average | 74.7 | 71.5 |

development in a manner analogous to glutathione. *Method A* was obviously unsuitable for reaction rate studies with pyridine and pyridine derivatives such as nicotinamide and pyridoxine since their reaction products would be expected to give colored solutions in the presence of alkali. Finally, the extremely high concentrations of substrates required for studies on macromolecules, and kinetic measurements at low pH values, interfered with color development making it necessary to employ *Method B*.

REACTION RATES WITH SMALL MOLECULES

Glycolic acid, acetyl-DL-alanine, and acetamide when present in 1,000 molar equivalents failed to react with the *s*-triazine at rates appreciably faster than its hydrolysis rate, so evidently carboxyl, aliphatic hydroxyl and amide groups as well as the peptide linkage as exemplified by acetyl-alanine are not involved as primary reaction centers. Purines, pyrimidines, and their derivatives such as adenine, ATP, cytosine and thymine were found to be essentially unreactive. Coenzyme I showed some reactivity, although the rate was too slow to permit accurate measurement. Indole-3-acetic acid did not react, so the primary amino group is probably the only center involved in the reactivity of tryptophan.

Aspartic acid is the least reactive compound on which quantitative measurements were made, so for convenience it was assigned a reactivity

of unity and the other compounds compared to it as a standard (Table II). In general organic acids containing only primary amino, hydroxyl, and aromatic groups reacted 2 to 10 times more rapidly than aspartic acid depending on the nature and position of the substituents. The amides as represented by asparagine and glutamine reacted several times more rapidly than the acids from which they are derived, although the amide group in acetamide had been shown to be unreactive.

TABLE II
APPARENT REACTIVITY OF AMINO ACIDS, PEPTIDES, AND CO-FACTORS WITH
2,4-DICHLORO-6-(*o*-CHLOROANILINO)-*s*-TRIAZINE AT 29° C. AND
PH 7.0 COMPARED TO ASPARTIC ACID = 1.0

| Compound | Method used | Mols compound/mol triazine | Half time (minutes) | Relative reactivity |
|-------------------------------------|-------------|----------------------------|---------------------|---------------------|
| Aspartic acid | A | 2,000 | 274 | 1.0 |
| α -Alanine | A | 1,000 | 312 | 1.8 |
| β -Alanine | A | 1,000 | 247 | 2.2 |
| Glutamic acid | A | 1,000 | 238 | 2.3 |
| Leucine | A | 1,000 | 164 | 3.3 |
| Thiamine | B | 1,000 | 136 | 3.9 |
| Serine | A | 1,000 | 122 | 4.3 |
| Asparagine | A | 1,000 | 111 | 4.8 |
| Valine | A | 1,000 | 107 | 5.0 |
| Glutamine | A | 1,000 | 105 | 5.1 |
| Isoleucine | A | 1,000 | 98 | 5.4 |
| Threonine | A | 1,000 | 90 | 5.9 |
| Methionine | A | 1,000 | 84 | 6.3 |
| Arginine | A | 1,000 | 83 | 6.4 |
| Histidine | A | 1,000 | 79 | 6.8 |
| Glycine | A | 1,000 | 73 | 7.3 |
| Phenylalanine | A | 1,000 | 70 | 7.6 |
| Tryptophan | A | 500 | 124 | 8.6 |
| Lysine | A | 1,000 | 58 | 9.2 |
| Alanylalanine | A | 1,000 | 53 | 10 |
| Alanylphenylalanine | A | 1,000 | 45 | 12 |
| Alanylglycylglycine | A | 1,000 | 40 | 13 |
| Leucylglycine | A | 1,000 | 35 | 15 |
| Nicotinamide | B | 1,000 | 33 | 16 |
| Taurine | A | 1,000 | 33 | 16 |
| Leucylglycylglycine | A | 1,000 | 23 | 23 |
| <i>p</i> -Hydroxybenzoic acid | B | 200 | 100 | 27 |
| Glycylalanine | A | 100 | 151 | 36 |
| Glycylleucine | A | 100 | 148 | 36 |
| Glycylglycine | A | 100 | 142 | 38 |
| Glycylglycylglycine | A | 100 | 126 | 43 |
| <i>p</i> -Hydroxyphenoxyacetic acid | B | 200 | 61 | 45 |
| Pyridoxine | B | 100 | 103 | 53 |
| Tyrosine | B | 100 | 97 | 55 |
| Proline | A | 100 | 85 | 63 |
| Pyridoxamine | B | 100 | 63 | 87 |
| Pyridine | B | 100 | 31 | 177 |
| Hydroxyproline | A | 100 | 29 | 185 |
| <i>p</i> -Aminobenzoic acid | A | 100 | 23 | 230 |
| Cysteine | B | 10 | 55 | 1000 |
| Glutathione | B | 10 | 55 | 1000 |

Compounds containing a secondary amino group or a pyridine ring were much more reactive. For example, proline and hydroxyproline were found to be 63 and 185 times more reactive than aspartic acid, while nicotinamide, pyridoxine and pyridoxamine were 16, 53, and 87 times more reactive in that order. The most reactive compound in which an amino group served as the only functional group was *p*-aminobenzoic acid with a relative reactivity of 230. This was exceeded only by cysteine and glutathione with relative reactivities of 1,000. In these latter two cases reactivity was tentatively ascribed to the sulfhydryl group since methionine is only 6 times more reactive than aspartic acid, and its rate is not measurable at the low concentrations required for the thiol derivatives. However, it is probable that a small part of the observed reactivity was with the α -amino group.

The most interesting development was the finding that peptide bond formation increases the reactivity of the terminal amino group. Thus glycylglycine was found to be 5 times more reactive than glycine, and glycylglycylglycine 6 times more reactive. This sequence of events was observed in peptides terminating with α -alanine and leucine residues (Table II) and so must be of general occurrence. It is unlikely that reaction can occur at the peptide bond since both acetylalanine and acetamide are unreactive.

These investigations show that secondary amines, pyridine derivatives, and particularly *p*-aminobenzoic acid might serve as receptor sites for toxicants which act primarily as alkylating agents, in addition to the more usual candidates, cysteine and glutathione.

EFFECT OF pK'

Many of the anomalies in the observed reaction rate constants can be resolved if it is assumed that only the $R-NH_2$ and $R-S^-$ groups in the equilibrium mixtures



are capable of reacting. In this event the usual second order rate equation (1) becomes

$$k' = \frac{2.303(K' + [H^+])}{t(a - b)K'} \log \frac{b(a - x)}{a(b - x)} \quad (5)$$

where K' is the dissociation constant of the functional group of the compound, a the molar concentration of the compound, b the concentration of triazine, and x the amount of triazine reacted at time t .

Activity coefficients and thermodynamic dissociation constants were

not used in these calculations since insufficient information was available for a uniform treatment. It must also be taken into account that the values for pK' found in the literature were obtained under different conditions of temperature and ionic strength than were used in these experiments. Even with these limitations the larger differences in intrinsic reactivity are readily apparent.

This is illustrated by the fact that when these corrections are made, the compounds (Table II) can be further divided into groups which show some correlation between structure and reactivity. For example, the substitution of a methyl group on the α -carbon atom of glycine to form α -alanine decreases reactivity by about two-thirds (Table III). This is

TABLE III
EFFECT OF POSITION AND SUBSTITUENTS ON THE REACTIVITY OF AMINO GROUPS WITH
2,4-DICHLORO-6-(*o*-CHLOROANILINO)-*s*-TRIAZINE AT 29° C. AND PH 7.0

| Compound | Apparent rate constant ($k \times 10^3$) | pK' | Corrected rate constant (k') |
|-----------------------------|---|-------|-------------------------------------|
| Glycine | 8.7 | 9.60 | 3.5 |
| α -Alanine | 2.1 | 9.69 | 1.0 |
| β -Alanine | 2.6 | 10.19 | 4.0 |
| Taurine | 19 | 8.74 | 1.1 |
| Proline | 76 | 10.6 | 300 |
| Hydroxyproline | 220 | 9.73 | 118 |
| Pyridine | 206 | 5.19 | 2.1 |
| <i>p</i> -Aminobenzoic acid | 273 | 4.92 | 0.28 |

probably a steric effect since alkyl substitution would be expected to increase the nucleophilicity of the amino group. This appears to be substantiated by the fact that shifting the amino group to the β -carbon atom as in β -alanine results in reactivity at least as high as that of glycine. However, when the carboxyl group is replaced by the more highly electrophilic sulfonic acid group as in taurine, reactivity drops sharply. These relationships are not apparent in the uncorrected data.

The secondary amines, proline and hydroxyproline, are much more reactive than the primary amines on either basis, which would be expected. However, the reactivity of proline is partly obscured by its high value for pK'_b . On the other hand, pyridine which appears to be about 180 times more reactive than aspartic acid is only about 4 to 5 times as reactive when the correction for pK'_b is made. The most drastic change occurs in the case of *p*-aminobenzoic acid. On the basis of observed rate it is the third most reactive compound investigated, while the corrected rate constant is one of the lowest in the group of compounds examined. This would be expected, since the aromatic ring should decrease both the basicity and nucleophilicity of the amino group.

Reactivity also varies in a uniform way when the substituent on the

TABLE IV

EFFECT OF SUBSTITUENTS ON THE α -CARBON ATOM ON THE REACTIVITY OF MONOCARBOXYLIC AMINO ACIDS WITH 2,4-DICHLORO-6-(*o*-CHLOROANILINO)-*s*-TRIAZINE AT 29° C. AND PH 7.0

| Amino acid | Substituent | Apparent rate constant ($k \times 10^3$) | pK' | Corrected rate constant (k') |
|-------------------|---|---|-------|-------------------------------------|
| Threonine | $\text{CH}_3\text{CHOH—}$ | 7.1 | 9.00 | 0.72 |
| Serine | $\text{HO—CH}_2\text{—}$ | 5.2 | 9.15 | 0.74 |
| α -Alanine | $\text{CH}_3\text{—}$ | 2.1 | 9.69 | 1.0 |
| Phenylalanine | $\text{C}_6\text{H}_5\text{CH}_2\text{—}$ | 9.1 | 9.13 | 1.2 |
| Methionine | $\text{CH}_3\text{SCH}_2\text{CH}_2\text{—}$ | 7.6 | 9.21 | 1.2 |
| Leucine | $(\text{CH}_3)_2\text{CH—CH}_2\text{—}$ | 3.9 | 9.60 | 1.6 |
| Valine | $(\text{CH}_3)_2\text{CH—}$ | 6.0 | 9.62 | 2.5 |
| Isoleucine | $(\text{CH}_3)(\text{C}_2\text{H}_5)\text{CH—}$ | 6.5 | 9.68 | 3.1 |

α -carbon atom of a glycine residue is varied (Table IV). Thus both serine and threonine which contain electron-attracting hydroxyl groups are less reactive than α -alanine, while phenylalanine and methionine have about the same order of reactivity. However, reactivity increases rapidly as the number of alkyl groups and their proximity to the α -carbon atom increases in the series leucine, valine, and isoleucine. The last named compound is almost as reactive as glycine indicating that the electron-releasing properties of the alkyl groups have counteracted the steric effect produced by α -substitution, and the increase in molecular size.

The relation between aspartic and glutamic acids and their amides are more reasonable on the revised basis (Table V). Originally the amides appeared to be about 3 to 6 times more reactive than the free acids which did not seem likely from a structural point of view. The corrected values agree more closely, with the amides being somewhat less reactive than the free acids. Blocking one of the carboxyl groups serves to decrease the concentration of unreactive *zwitterion* as indicated by the lower values for pK'_b .

Equation (5) has been most successful in explaining the reactivities of the peptides in relation to the reactivities of the amino acids comprising

TABLE V

EFFECT OF AMIDE FORMATION ON THE REACTIVITIES OF AMINO ACIDS CONTAINING TWO CARBOXYL GROUPS WITH 2,4-DICHLORO-6-(*o*-CHLOROANILINO)-*s*-TRIAZINE AT 29° C. AND PH 7.0

| Compound | Substituent on α -carbon atom | Apparent rate constant ($k \times 10^3$) | pK' | Corrected rate constant (k') |
|---------------|---|---|-------|-------------------------------------|
| Aspartic acid | $\text{HOOC—CH}_2\text{—}$ | 1.2 | 9.60 | 0.47 |
| Asparagine | $\text{NH}_2\text{CO—CH}_2\text{—}$ | 5.8 | 8.80 | 0.37 |
| Glutamic acid | $\text{HOOC—CH}_2\text{—CH}_2\text{—}$ | 2.7 | 9.67 | 1.3 |
| Glutamine | $\text{NH}_2\text{CO—CH}_2\text{—CH}_2\text{—}$ | 6.1 | 9.13 | 0.82 |

their terminal residues. Increases in size and complexity would be expected to decrease reactivity by reducing the probability of a successful collision between reacting molecules. There is no apparent reason why amide or peptide bond formation should activate the α -amino group, so the observation that peptides reacted much more rapidly than the free amino acids seemed inconsistent. However, peptide bond formation increases the distance between the terminal amino and carboxyl groups and so increases the *amount* of free base at the expense of the *zwitterion*. Because of this, the apparent reaction rate increases. The largest drop in pK'_b occurs with the establishment of the first bond, and thereafter the

TABLE VI
COMPARATIVE REACTIVITIES OF AMINO ACIDS AND PEPTIDES WITH 2,4-DICHLORO-6-(*o*-CHLOROANILINO)-S-TRIAZINE AT 29° C. AND PH 7.0

| Compound | Apparent rate constant ($k \times 10^3$) | pK'_b | Corrected rate constant (k') |
|---------------------|---|---------|-------------------------------------|
| α -Alanine | 2.1 | 9.69 | 1.0 |
| Alanylalanine | 12 | 8.42 | 0.33 |
| Alanylphenylalanine | 14 | 8.35 | 0.33 |
| Alanylglycylglycine | 16 | 8.15 | 0.24 |
| Leucine | 3.9 | 9.60 | 1.6 |
| Leucylglycine | 18 | 8.18 | 0.30 |
| Leucylglycylglycine | 28 | 7.97 | 0.29 |
| Glycine | 8.7 | 9.60 | 3.5 |
| Glycylleucine | 43 | 8.29 | 0.88 |
| Glycylalanine | 43 | 8.25 | 0.80 |
| Glycylglycine | 45 | 8.13 | 0.66 |
| Glycylglycylglycine | 51 | 7.91 | 0.46 |
| Cysteine | 1,200 | — | * |
| Glutathione | 1,200 | — | — |

* This value has been omitted since some uncertainty exists concerning the assignment of the pK' values of cysteine (2,7,9).

decreases are relatively small (8, p. 85). When this correction is applied, reactivity decreases rather than increases in the series of peptides terminated by α -alanine, leucine, and glycine (Table VI). In this connection it is interesting to note that the largest drop in intrinsic reactivity occurs with the establishment of the first peptide linkage and thereafter decreases. This indicates that replacement of a free carboxyl group with a peptide linkage may have a more pronounced effect on decreasing chemical activity than the change in molecular size. This is given some confirmation by the previous observation (Table V) that amides are somewhat less reactive than free acids when corrections are made for their dissociation constants.

A number of materials were included in this investigation which are

difficult to classify under a definite category since some of them may have more than one functional group capable of reacting with the triazine (Table VII). For example, lysine could react at both the *alpha* and terminal amino groups, arginine at the amino and guanidino groups, and pyridoxamine at the amino and hydroxyl groups or at the ring nitrogen. It would be impossible to assign rates to these individual reactions without a knowledge of the distribution of the products so values for κ were calculated which probably represent the sum of the individual rate constants. Some of these compounds such as tryptophan could be included in Table IV since the indole ring in indole-3-acetic acid is not reactive thus making it likely that most of the triazine combines with the α -amino group. The same reasoning cannot be applied to tyrosine since the hydroxyl groups of *p*-hydroxybenzoic and *p*-hydroxyphenoxyacetic acids (Table II) react rapidly. It must be noted that phenylalanine is only 9 times more reactive than aspartic acid, while tyrosine is 66 times more reactive and the values for pK' for the two compounds are almost identical. A hydroxyl group substituted on an aromatic ring can increase electron density at the *para* position through an electromeric shift but it is difficult to see how this could be transmitted through a methylene bridge effectively enough to cause a 6-fold increase in rate.

TABLE VII

APPARENT REACTION RATE CONSTANTS FOR MISCELLANEOUS AMINO ACIDS AND CO-FACTORS FOR WHICH THE NUMBER OF FUNCTIONAL GROUPS IS UNKNOWN

| Compound | Potential reaction sites | Apparent rate constant ($\kappa \times 10^3$) |
|--------------|--|--|
| Histidine | α -Amino Imidazole NH | 8.1 |
| Tryptophan | α -Amino Indole NH | 10 |
| Tyrosine | α -Amino Phenolic OH | 66 |
| Lysine | α -Amino ϵ -Amino | 11 |
| Arginine | α -Amino δ -Guanidino | 7.7 |
| Thiamine | Primary amino, heterocyclic nitrogen | 4.7 |
| Pyridoxamine | CH ₂ OH, CH ₂ NH ₂ Phenolic OH Tertiary N | 69 |
| Pyridoxine | CH ₂ OH Phenolic OH Tertiary N | 76 |

RELATION BETWEEN BASICITY AND NUCLEOPHILICITY

The basicity of an amino acid is described in part by its value for pK'_b since this is a measure of the affinity of the amino group for a proton. On the other hand the nucleophilicity of the group, or its tendency to participate in displacement reactions at positively charged centers, is represented by its second order velocity constant. Since both of these values contain elements of the affinity of the group for a positive charge some correlation between them is likely.

This would not be apparent in an assembly of compounds selected at random, since these values can be modified by other factors. For example, the ionization of an amino group is repressed by the presence of a carboxyl group in the molecule, even though its inductive effect leads to a net decrease in pK' , so that a compound such as glycine with a pK'_b of 9.60 is less dissociated than its methyl ester with a pK'_b of 7.61 (8, p. 99). This effect diminishes as the distance between the two groups increases. This is evident in peptides terminating in α -alanine, leucine, and glycine residues (Table VI) where it is seen that the pK'_b values for the peptides are always lower than for the corresponding amino acids. Conversely, the presence of a second carboxyl group in the molecule results in a further repression of ionization. Thus the pK'_b values for glutamic and aspartic acids are considerably higher than for their corresponding amides (Table V). It is also evident that different functional groups cannot be compared with one another. Thus the primary amino group in glycine, the secondary amino group in proline, and the sulfhydryl group in cysteine vary enormously in reactivity, and this obviously is not correlated with pK'_b .

In order to minimize these conflicting effects this analysis was confined to monofunctional compounds containing a single carboxyl group with a primary amino group substituted on the carbon atom *alpha* to it. This automatically includes glycine, α -alanine, threonine, serine, phenylalanine, methionine, leucine, valine, isoleucine, asparagine and glutamine. Histidine and tryptophan were also included since it is unlikely that the imidazole and indole rings react at appreciable rates. On preliminary examination, it was found that α -alanine was not a member of the group. Apparently substitution of a methyl group on the α -carbon of glycine is able to reduce reactivity through steric effects without influencing pK'_b . In view of this, the values for it were excluded from the calculations.

To determine whether the relation between corrected velocity constant (k'), and dissociation constant (K'_b) is exponential, the regression of $\log k'$ on pK'^r was computed by the method of least squares. This equation reduces to

$$k' = \frac{1}{1.86 \times 10^8} \left(\frac{1}{K'_b} \right)^{0.9} \quad (6)$$

which indicates that the rate constant is inversely proportional to the dissociation constant to a first approximation. The correlation coefficient was found to be 0.93 which is highly significant at the 1 per cent level.

Considering the heterogeneity of the data, the exponent of (6) approaches unity as closely as can be expected, so the regression of k' on $1/K'$ was computed with the result that

$$k' = \frac{6.7 \times 10^{-10}}{K'_b} + 0.04 \quad (7)$$

Equation (7) as well as the regression curve (Fig. 2) shows that the intercept is close to zero. This means that if the basicity of the amino group could be reduced to a point where dissociation is complete, the ability of the compound to take part in nucleophilic reactions would vanish. When $K'_b = 0$, the intrinsic reactivity should be infinite. Paradoxically, the observed rates at both extremes should be zero since in the latter case none of the compound would be available in reactive form.

The correlation coefficient using the parameters required to obtain equation (7) is 0.87. This is also highly significant at the 1 per cent level. Some advantage is gained using the exponential form, but in view of the disperse data it is probable that this is more apparent than real. In any

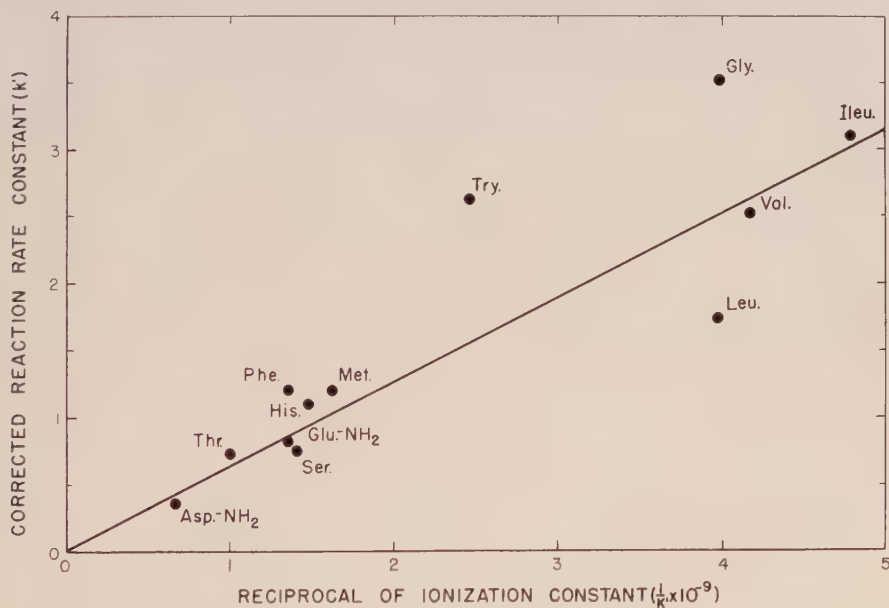


FIGURE 2. Relationship between corrected rate constant and reciprocal of dissociation constant for α -amino acids having a single carboxyl group.

event it is obvious that the relation between k' and pK'_b is not quantitative even when the compounds are selected with regard to structure. This is to be expected, since reaction rates may be affected by factors other than nucleophilicity, so even with the imposed restrictions, the character of the substituent groups probably varies too widely to expect homogeneity. A more definitive test could be obtained by evaluation of a series such as



where $X = CH_3, OH, OCH_3, CN$, etc., so that the electronegativity of the substituent could be varied without major changes in other properties.

However, the significant correlation obtained between basicity and nucleophilicity serves to support the corrections made in the rate constants by use of equation (5), for when the observed constants are plotted against $1/K'_b$ a scatter diagram is obtained (Fig. 3). A procedure which transforms data such as these to a relation which is significant at the 1 per cent level probably has some validity.

EFFECT OF pH

Equation (5) contains a term for the hydrogen-ion concentration of the solution as well as the dissociation constant of the substrate. According to

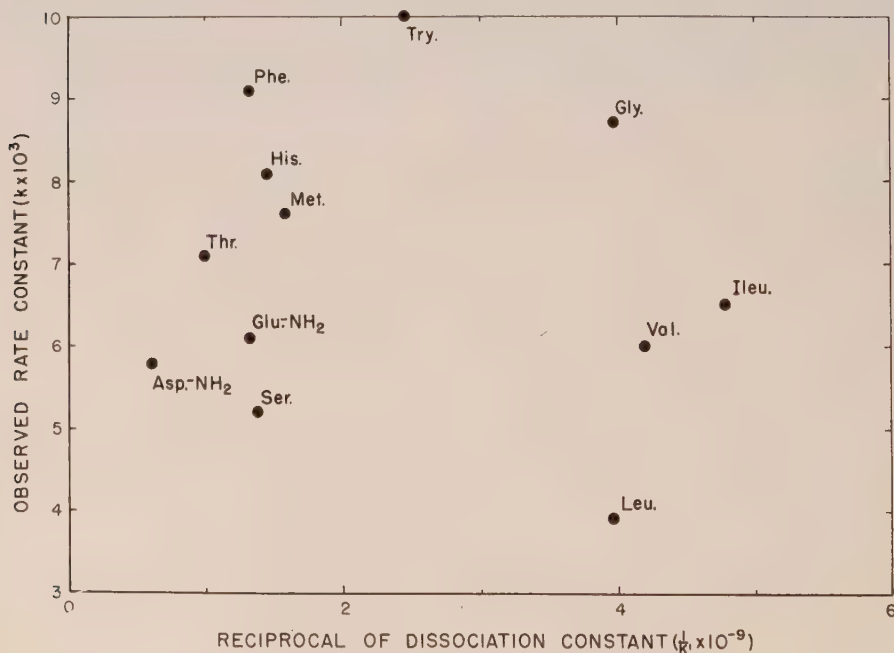


FIGURE 3. Scatter diagram showing lack of correlation between reciprocal of dissociation constant and observed rate constant.

TABLE VIII

EFFECT OF pH ON THE REACTION RATE OF 2,4-DICHLORO-6-(*o*-CHLOROANILINO)-5-TRIAZINE WITH GLYCINE AT 30° C.

| pH | Mols substrate/ mol triazine | Half time (minutes) | Apparent rate constant (<i>k</i>) | Corrected rate constant (<i>k'</i>) |
|-----|---------------------------------|------------------------|---|---|
| 5.0 | 1 × 10 ⁵ | 58 | 1.1 × 10 ⁻⁴ | 4.4 |
| 5.5 | 3.3 × 10 ⁴ | 61 | 3.2 × 10 ⁻⁴ | 4.2 |
| 6.0 | 1 × 10 ⁴ | 58 | 1.1 × 10 ⁻³ | 4.4 |
| 6.5 | 3.3 × 10 ³ | 54 | 3.6 × 10 ⁻³ | 4.7 |
| 7.0 | 1 × 10 ³ | 62 | 1.0 × 10 ⁻² | 4.1 |

this, the observed reaction rates should be lowered drastically when pH is reduced. This was investigated for the cases of cysteine and glycine.

At pH 5.0 and 10³ equivalents of glycine the reaction would take place too slowly to permit accurate measurement because of complicating factors such as hydrolysis of the triazine and decomposition of the substrate by bacterial contamination. As an alternate method the reaction time was held approximately constant by increasing the concentration of substrate. This procedure is not entirely satisfactory since activity coefficients would not remain constant over a 100-fold concentration range. However, the results were sufficiently consistent to substantiate equation (5). Thus the corrected rate constant for glycine (Table VIII) remained fairly constant from pH 5 to pH 7 while the apparent rate constant increased from 10⁻⁴ to 10⁻². According to these figures only about 0.0025 per cent of the substrate is available in reactive form at pH 5.

Similar results were obtained with cysteine (Table IX). The corrected coefficient remained approximately constant while the apparent rate shifted from 0.017 to 1.7 when the pH was increased from 5 to 7. When the logarithms of the apparent rate constants were plotted against pH values, the curves obtained for cysteine and glycine were parallel indicating quantitative correspondence with equation (5) for both the amino and sulfhydryl groups (Fig. 4). This leads to the generalization that the ap-

TABLE IX

EFFECT OF pH ON THE REACTION RATE OF 2,4-DICHLORO-6-(*o*-CHLOROANILINO)-5-TRIAZINE WITH CYSTEINE AT 30° C.

| pH | Mols substrate/ mol triazine | Half time (minutes) | Apparent rate constant (<i>k</i>) | Corrected rate constant* |
|-----|---------------------------------|------------------------|--|-----------------------------|
| 5.0 | 1 × 10 ³ | 38 | 0.017 | 1.0 |
| 5.5 | 3.3 × 10 ² | 38 | 0.051 | 1.0 |
| 6.0 | 1 × 10 ² | 32 | 0.20 | 1.2 |
| 6.5 | 33 | 33 | 0.59 | 1.1 |
| 7.0 | 10 | 39 | 1.7 | 1.0 |

* Adjusted to unity at pH 7.0 since the value for *pK'* is uncertain (2,7,9).

parent reactivities of the functional groups of amino acids decrease 10-fold for each unit decrease in pH.

The good agreement obtained between the calculated and observed values for reaction rate constants as a function of hydrogen-ion concentration strengthens the case for correcting the observed values obtained on amino acids and peptides for dissociation. However, it could be argued that the rates are higher at higher pH values because of general basic catalysis. If this were the case the reaction would be of the S_N1 type (28, p. 200) in which the addition of a hydroxyl group to the triazine molecule is the

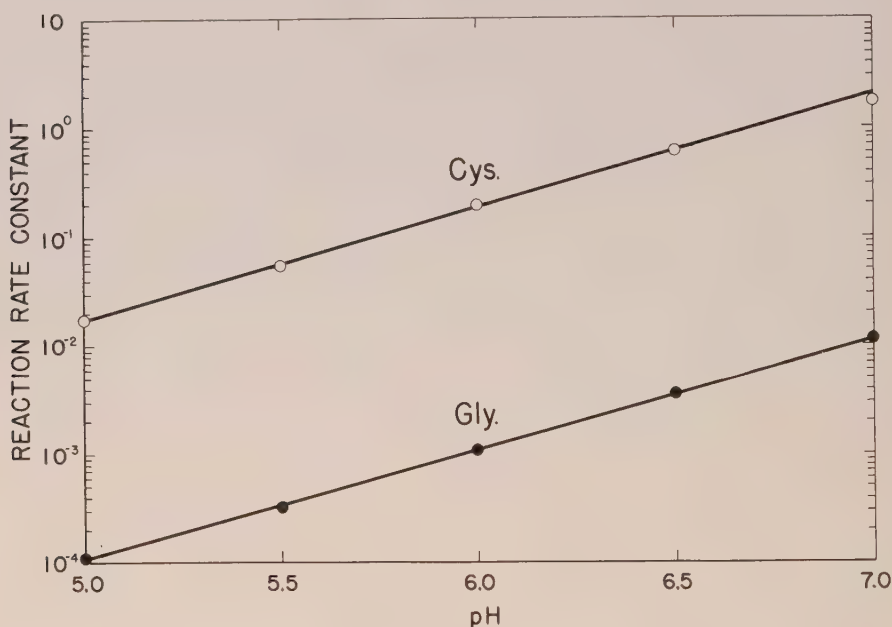


FIGURE 4. Effect of pH on the apparent reaction rate constant of 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine with cysteine, and glycine.

rate-determining step. This would imply that the reaction rate should be independent of the nature of the substrate which is obviously not the case. In view of the evidence it seems more reasonable to conclude that the reaction is of the S_N2 type and ascribe the decrease in reactivity on reducing pH to repression of the ionization of the functional group by mass action.

REACTION RATES WITH MACROMOLECULES

Since the *s*-triazines might inactivate enzymes or change the physico-chemical characteristics of proteins through combination with their side

chain and terminal groups, the reaction rates of 2,4-dichloro-6-(*o*-chloro-anilino)-*s*-triazine with a number of macromolecules were determined. The materials used in these investigations were selected primarily on grounds of purity and availability regardless of their origin. Since the number, and probably the reactivities, of the functional groups varied from compound to compound, concentrations were adjusted on a weight rather than on a molar basis. The amount of residual triazines could not be determined in the presence of the substrates, so in all cases the extraction procedure (*Method B*) was used.

In most cases the concentrations of substrates required to cause the reactions to proceed at convenient rates were rather large in comparison to the amounts of amino acids that were used, but it must be taken into

TABLE X
REACTIVITY OF 2,4-DICHLORO-6-(*o*-CHLOROANILINO)-*s*-TRIAZINE WITH LARGE MOLECULES

| Material | Concentration (mg. per ml.) | Half time (minutes) | Relative reactivity egg albumin = 1 |
|------------------------|--------------------------------|------------------------|--|
| Edestin | 5 | > 10 ³ | ca. 0 |
| RNA | 10 | ca. 360 | 0.4 |
| DNA | 10 | 130 | 1 |
| Egg albumin | 10 | 130 | 1 |
| Intestinal phosphatase | 10 | 70 | 2 |
| Pepsin | 10 | 50 | 3 |
| Lysozyme | 10 | 50 | 3 |
| Malt diastase | 10 | 45 | 3 |
| Pancreatic amylopsin | 10 | 40 | 3 |
| Trypsinogen | 2.4 | 100 | 5 |
| Urease | 10 | 20 | 7 |

consideration that only the terminal amino groups and the side chains derived from cysteine, lysine, arginine, and probably tyrosine residues are capable of taking part in the reactions. In the presence of 1 per cent egg albumin the half time of the triazine at a concentration of 5 μ g. per ml. was 130 minutes. This compound was used as a standard and the reactivities of other macromolecules calculated with reference to it (Table X).

Amino acid analyses (23, p. 217) show that 58 equivalents of SH, 250 equivalents of ϵ -NH₂, and 188 equivalents of guanidino groups were present so evidently the functional groups of egg albumin are not present in a highly reactive form. However, edestin at a concentration corresponding to 5.5 equivalents of terminal NH₂ (glycine), and 11, 44, and 264 equivalents of SH, NH₂, and guanidino respectively failed to react at a measurable rate. Intestinal phosphatase, pepsin, lysozyme, malt diastase, pancreatic amylopsin, trypsinogen, and urease reacted from 2 to 7 times faster than egg albumin. The relatively high reactivity of lysozyme is interesting since amino acid analysis (23, p. 219) has shown that cysteine residues are

absent. This would imply that most of the reaction took place with terminal amino, ϵ -NH₂ (222 equivalents) and guanidino (407 equivalents) groups unless it could be shown that some of the cystine residues were present in reduced form.

It is also interesting to note that RNA and DNA showed some reactivity with DNA being the more active. This was unexpected, since the purine and pyrimidines which were examined did not cause the disappearance of the triazine at measurable rates, and the ribose and phosphate residues do not contain groups which would be expected to serve as reaction centers. However, these compounds were commercial chemicals and it is possible that the observed rates may have been due to the presence of impurities.

RELATED COMPOUNDS

The dichloro-*s*-triazines are members of a large class of compounds containing active halogen atoms which are capable of undergoing replacement reactions with amino and sulfhydryl groups. Other representatives are iodoacetate, 2,4-dinitrofluorobenzene, bis(2-chloroethyl) sulfide [mustard gas], chloropicrin, chloroacetone, chloroacetophenone (1), phosgene, etc. Their biological potentials are probably regulated by their reaction rates and polarities in relation to the environments in which they must operate, and the characteristics of the species against which they must perform.

The reactions of iodoacetate have been studied extensively because of its widespread use as an enzyme inhibitor. Michaelis and Schubert (20) have shown that it reacts with sulfhydryl groups to form thioethers, but Hellström (14) claims that sulfides and disulfides are formed with thio-glycolic acid. Smythe (32), contrary to the data presented in this paper, has shown that it reacts with cysteine more rapidly than with glutathione. However, he demonstrated that it reacts considerably more rapidly at pH 7.1 than at 6.1 which supports the view that the anion must be present. Brdicka (3) studied the reaction kinetics of glycine with iodoacetate in the range pH 8.18 to 13.17 and came to the conclusion that the NH₃⁺ group could not take part in the reaction. He proposes an equation similar to (6) except that it contains additional terms for the alkaline hydrolysis of the acetate and takes into account the fact that two molecules of iodoacetate react with each amino group. He has also shown that velocity constants increase with ionic strength, thus showing positive salt catalysis.

The reactions of bis(2-chloroethyl) sulfide have been studied extensively because of its use as a war gas (13), while Sanger (30) and others have used 2,4-dinitrofluorobenzene as a reagent for determining the sequence of amino acids in peptide chains. The literature on the reactions of active halogen compounds with biological systems is so large that a complete review would be beyond the scope of this paper.

GENERAL DISCUSSION

These present investigations had a dual purpose; first to obtain a better understanding of the reactivities of amino and sulfhydryl groups in relation to molecular structure, and secondly to determine which compounds in living organisms are likely to be most reactive.

Before structure could be correlated with reactivity it was necessary to correct the observed rate constants for the dissociation of the functional groups. Once this was done the results conformed satisfactorily with the electronic theories of organic chemistry as developed by Lapworth, Ingold, Robinson and others. It cannot be overemphasized that dissociation and reactivity are interrelated so that the effect of any substituent group must be interpreted in terms of both factors. A carboxyl group probably decreases the intrinsic reactivity of an adjacent amino group by attracting electrons away from it, but this is counterbalanced by the fact that the basicity of the group is reduced so that more of it can ionize to the reactive form. Thus the pK'_b for ethylamine is 10.67, while the pK'_b for glycine is 9.60 indicating a 10-fold increase in the amount of free NH_2 on substituting a carboxyl for a methyl group.

At this point a third factor must be introduced since a carboxyl group next to an amino group tends to repress its ionization and hence its reactivity by the creation of a micro-environment which behaves as if it were more acidic than the main body of the solution. This effect is reduced as the distance between the two groups is increased. Thus pK'_b decreases progressively from 9.60 to 7.60 in the series, glycine . . . hexaglycine (8, p. 85). The largest drop occurs on the establishment of the first linkage. Part of this may be caused by differences in the electron-attracting properties of the carboxyl and amide groups, but it seems certain that spatial separation is also involved. Another observation which supports this is the fact that ionization of an α -amino group is increased when the carboxyl group is masked. Thus the pK'_b of the methyl ester of glycine is 7.73 compared to 9.60 for glycine indicating that its reactivity should be much higher if the electrophilicities of the carboxyl and ester groups have the same order of magnitude.

Thus it is possible that the reactivities of amino acids and simple peptides might be quite different from those of proteins where there are fewer free carboxyl groups and the long chain peptides are intimately associated with one another. In such complex molecules regions of proton deficiency might occur where functional groups could exist in a more completely ionized state than occur in amino acids. The possible gains from such situations are not inconsequential when it is considered that only 0.25 per cent of the reaction potential of glycine is utilized at pH 7.0.

From the standpoint of applications to living organisms the relation between pH, pK' and reaction rate again appears to be the most useful

observation arising from these studies, since it may ultimately prove to be one of the reasons for species specificity. Many fungi thrive on acid media, and it is not unlikely that their internal pH values would be on the acid side. Since reactivity decreases 10-fold for each unit decrease in pH, it would be expected that a fungus species whose normal internal pH was 5 to 6 would be less susceptible to toxicants of this class than species with a pH range of 6 to 7, assuming of course equivalent uptake of the chemical and viability of the spores.

With regard to sites of action the potential importance of glutathione is once more emphasized because of its high reactivity and the fact that it occurs in relatively large amounts in almost all plant and animal cells (15, 34, p. 46). However, thioctic acid, coenzyme A, and 2-mercaptoethylamine might have been found to be equally reactive had rate measurements been made on them.

The role of glutathione in metabolism has never been satisfactorily established. It is known to be a coenzyme for the conversion of methylglyoxal to lactic acid (27, p. 172) and as a prosthetic group of triose phosphate dehydrogenase. It is also known to take part in trans-peptidation reactions (12, p. 145) and may act as an intermediate in the synthesis of proteins. However, many workers believe that its primary function is to regulate the poise or oxidation-reduction potential of the cell, and maintain sulfhydryl-dependent enzymes in a reduced condition. In any case, it seems to be necessary for the germination of fungus spores since Wikberg (35) has obtained a glutathioneless mutant of *Ophiostoma multiannulatum* with an absolute requirement for glutathione.

However, experiments with conidia of *Neurospora sitophila* (4) indicate that it is not the only material affected since inhibition of germination is only partly reversed when spores which are subjected to the median lethal dosage of triazine are incubated in a solution containing glutathione. At a dosage which prevents all of the spores from germinating, none of them can be revived. This indicates that glutathione depletion is not the only factor involved in the toxicity of the *s*-triazines, although it may play an extremely important role in protecting spores against low doses of alkylating agents by acting as a detoxifying agent. This conclusion should be expected since the kinetic studies show that *p*-aminobenzoic acid, pyridoxine, pyridoxamine, and niacine are also reactive. Thus folic acid metabolism, transaminations, and biological reductions could be halted by the *s*-triazines. Proline and tyrosine reserves could be immobilized, and the protein molecules which catalyze biochemical reactions could be inactivated, so that the fungus spores would succumb to mass attacks on many of their vital functions.

This is a highly inefficient method of operation compared to compounds such as the antibiotics and diisopropyl fluorophosphate (19) which

appear more highly selective with regard to sites of action. Thus 1,500 μ g. of the *s*-triazine must be taken up from solution by 1 gram of conidia of *N. sitophila* to inhibit germination of 50 per cent of the spores (4, 5). By contrast Rowley *et al.* (29) have shown that penicillin is effective against staphylococci at concentrations within the bacteria of 2 μ g. per gram. In view of the data presented by Miller *et al.* (21, 22) on the large amounts of fungicides which must be taken up to prevent spore germination, the information obtained by Owens (24, 25) on their broad spectrum of anti-enzyme activity, and the failure of Leben *et al.* (17) to obtain resistant mutants, it seems unlikely that the fungitoxicans currently used in agriculture are highly selective in their modes of action.

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Note

USE OF SODIUM PEROXIDE FUSION FOR THE DETERMINATION OF FLUORIDES IN VEGETATION

R. MAVRODINEANU AND J. GWIRTSMAN

The procedure for analyzing fluorine in vegetation has been improved in recent years by the development of a new steam-distilling apparatus with electric heating (3) and the use of photoelectric end-point determination in the titration of fluorides with thorium nitrate (4).

In an effort to shorten the time required for the conversion of insoluble fluorine compounds into a soluble ionizable form, a direct fusion of the plant material with sodium peroxide (Na_2O_2) was carried out in a Parr bomb. The results showed that the average time required to complete a direct Na_2O_2 fusion is 30 minutes as compared with 6 hours necessary for the ashing with CaO used in the standard procedure.

The use of the Parr bomb for the decomposition of organic material has been known for a long time. A detailed review is given by Elving, Horton, and Willard (2), and a more recent paper (1) describes its use for the analysis of organic fluoro compounds. Apparently the sodium peroxide fusion has not been used previously for routine analysis of vegetation.

The principle of the Na_2O_2 fusion method using a bomb (Parr) is known (5, 6, 7, 8) and will not be repeated here, except for the kind of accelerator which can be used. Both potassium nitrate and potassium perchlorate are recommended, the latter being preferable, particularly if the nitrate content of the sample is high.

ANALYTICAL PROCEDURE AND PRELIMINARY RESULTS

A 0.5-g. sample of dried, finely-ground (in a Wiley cutting mill, 40 mesh), and homogenized plant material was placed in the fusion cup of a 22-ml. capacity Parr bomb. To this material were added 0.5 g. of sucrose and 0.5 g. of finely-ground accelerator together with a dipper (approximately 15 g.) of Na_2O_2 . The Parr bomb was then closed and its contents thoroughly mixed by rotating the bomb. Ignition was carried out as usual (6) and, after cooling, the resulting fused mixture was dissolved in 50 ml. of distilled fluorine-free water and transferred quantitatively to the steam-distilling apparatus (3).

Data in Table I show that comparable results covering a wide concentration range were obtained with the CaO ashing and Na_2O_2 fusion procedures. If similar results are obtained with other species, the Na_2O_2 fusion method may be employed more frequently in routine analyses.

TABLE I

RESULTS FROM TWO METHODS OF FLUORINE ANALYSIS: CaO ASHING VERSUS Na₂O₂ FUSION

| Species of leaves analyzed | CaO ashing, F p.p.m. | Na ₂ O ₂ fusion, F p.p.m. | Remarks |
|---|-------------------------|--|--|
| <i>Camellia japonica</i> L. Tea (<i>Camellia</i> sp.) | 790 100 | 795 102 | Natural plant Commercial Tetley tea |
| <i>Pygmaeothamnus zegheri</i> , Sample #1 | 12 | 12 | } Natural plant grown in Southern Rho- desia, Africa Plants fumigated continuously with HF for 7 days |
| “ “ “ #2 | 6 | 7 | |
| Cotton (<i>Gossypium hirsutum</i> L.) | 3188 | 3250 | |
| Alfalfa (<i>Medicago sativa</i> L.) | 427 | 468 | |

Parr fusion bombs of 22-ml. and 42-ml. capacity are now being used with and without electrical ignition. The 42-ml. fusion cup permits the use of 1 to 1.5-g. samples.

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RED PINE SCALE WITH SPECIAL REFERENCE TO ITS HOST PLANTS AND COLDHARDINESS

ALBERT HARTZELL¹

SUMMARY

The seasonal history of the recently discovered bark scale, *Matsucoccus resinosae* Bean & Godwin, on red pine, *Pinus resinosa* Ait., was investigated during the seasons of 1954-1956, at Yonkers, New York, and the various stages of the scale were observed and photographed. The insect was found to have two broods a year. Artificial infestation of 22 species of pine revealed three new host plants, namely, *Pinus tabulaeformis* Carr., *Pinus densiflora* Sieb. & Zucc., and *Pinus densiflora* var. *umbraculifera* Mayr., all of oriental origin. Two species of ladybird beetles, *Coccinella transversoguttata* Fald. and *Cleis picta* (Rand.) and a small hemipteron, *Xanotrachelliella inimica* D. & H., were found to be predaceous on the scale. These together with other natural enemies were not sufficiently abundant to effect any appreciable control.

A study of the coldhardiness of *Matsucoccus resinosae* on red pine under experimentally controlled conditions indicated that exposure to -10° to -29° F. for four to seven hours killed over 90 per cent of the overwintering stages of the scale and that repeated exposures of -10° F. killed 99 per cent. From this it may be deduced that rapid spread of the scale northward would be retarded, at least until a race is evolved that possesses greater coldhardiness than the present race of *Matsucoccus resinosae*.

INTRODUCTION

Prior to 1946, the only species of coccid of the genus *Matsucoccus* known to occur in northeastern United States was *Matsucoccus gallicolus* Morr., which was confined chiefly to pitch pine and scrub pine. In Easton, Connecticut in November 1946, Plumb (4) found a number of red pine (*Pinus resinosa* Ait.) heavily infested with a species of *Matsucoccus*. This proved to be a new species later described as *Matsucoccus resinosae* Bean & Godwin (2). By 1953 a survey of Connecticut showed that this infestation extended over an area of 60 sq. mi. This scale was first reported in New York State in 1950. An extensive survey made by the State of New York in 1953 showed an infested area of approximately 40 sq. mi. on Long Island, and scattered spot infestations in Westchester County, New York along the Hutchinson River Parkway and in Yonkers, Scarsdale and Tuckahoe.

Since a grove of about 100 mature red pine trees grown from seeds in the Boyce Thompson arboretum was found to be infested with *Matsucoccus resinosae*, a cooperative project between the Forest Service of the United States Department of Agriculture, the New York State Entomologist's

¹ With the technical assistance of William F. Munk and Gerald C. Johnson.
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Office of the State Museum and Science Service, the New York State Department of Conservation, and the Boyce Thompson Institute for Plant Research was begun in 1954 primarily to determine other hosts and the coldhardiness of this species of scale.

In the ten years that *Matsucoccus resinosae* has been known in the northeastern United States, it has become one of the most important pests of red pine plantations within its range. The distribution of the scale is definitely restricted at present. Thousands of trees, ranging from nursery stock to mature stands have already been killed (1) in Connecticut and Long Island.

DESCRIPTION

Matsucoccus resinosae adult females (2) are brownish red and wingless (Fig. 1 C). They range in length from 2 mm. to 5 mm. The body is roughly pear-shaped and is wrinkled. The preadult males resemble the adult females but are smaller and are approximately one-half the length of the females. Preadult males soon transform into true adults inside loosely woven, white, spindle-shaped cocoons.

The true adult males (Fig. 1 D) are two-winged and are midge-like in appearance. The minute, amber-yellow, oval-shaped eggs (Fig. 1 A) are laid in a loosely woven sac attached to the female. First-stage larvae (Fig. 1 B) resemble the adult female (Fig. 1 C) but are much smaller (0.4 by 0.2 mm.). The first-stage larvae transform into the intermediate legless stage, which is elliptical in shape and without antennae. In this stage the skin is tough and leathery, at first amber in color, later changing to grey-yellow with darker margins.

SEASONAL HISTORY

During the year, *Matsucoccus resinosae* has two well-defined generations. The first of the summer-generation eggs are laid in May. In Yonkers, New York, the first eggs of this generation were observed on May 26, 1954. The eggs of the fall generation are laid early in September and the adults emerge the following spring. The fall generation overwinters as partly grown, first-stage larvae which resume feeding in April and transform into the intermediate stage (Fig. 2 B). This stage develops rapidly and the preadult males appear about the first of May. They immediately enclose themselves in silken cocoons (Fig. 2 A) and transform into true adults, which emerge at approximately the same time as the adult females.

Within a day or two the fertilized females begin to lay eggs. The first-stage larvae appear about the first of June. After a short period of wandering around, the first-stage larvae (Fig. 1 B) settle down under bark scales and begin feeding, remaining in this position during the rest of the feeding period. These larvae develop into the intermediate stage by the middle of July. The adult males and females of the summer generation emerge

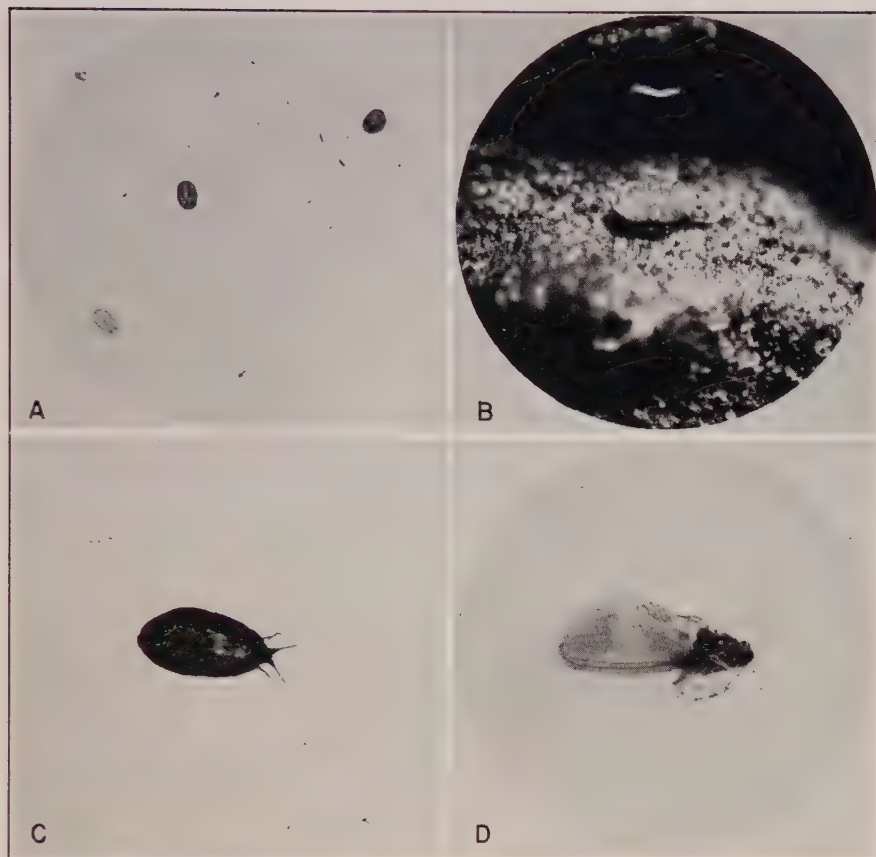


FIGURE 1. Red pine scale (*Matsucoccus resinosa*). (A) Eggs ($\times 25$). (B) Crawler ($\times 70$). (C) Female ($\times 7$). (D) Male ($\times 15$).

around the middle of August and lay the eggs that produce the overwintering generation. An overlapping of the two generations makes it difficult to determine the duration of the various stages.

HOSTS

Because of a considerable number of mature exotic species of seedling pine trees growing in the arboretum of the Institute at Yonkers, N. Y., the locality afforded an excellent opportunity to determine whether this species of scale is confined to a single species of pine, as was suspected, or was capable of establishing itself on other hosts.

During the growing seasons of 1954, 1955, and 1956 attempts were made to infest 22 species of pine in the arboretum with *Matsucoccus resinosa* scale by tying infested twigs of red pine to the outer branches during

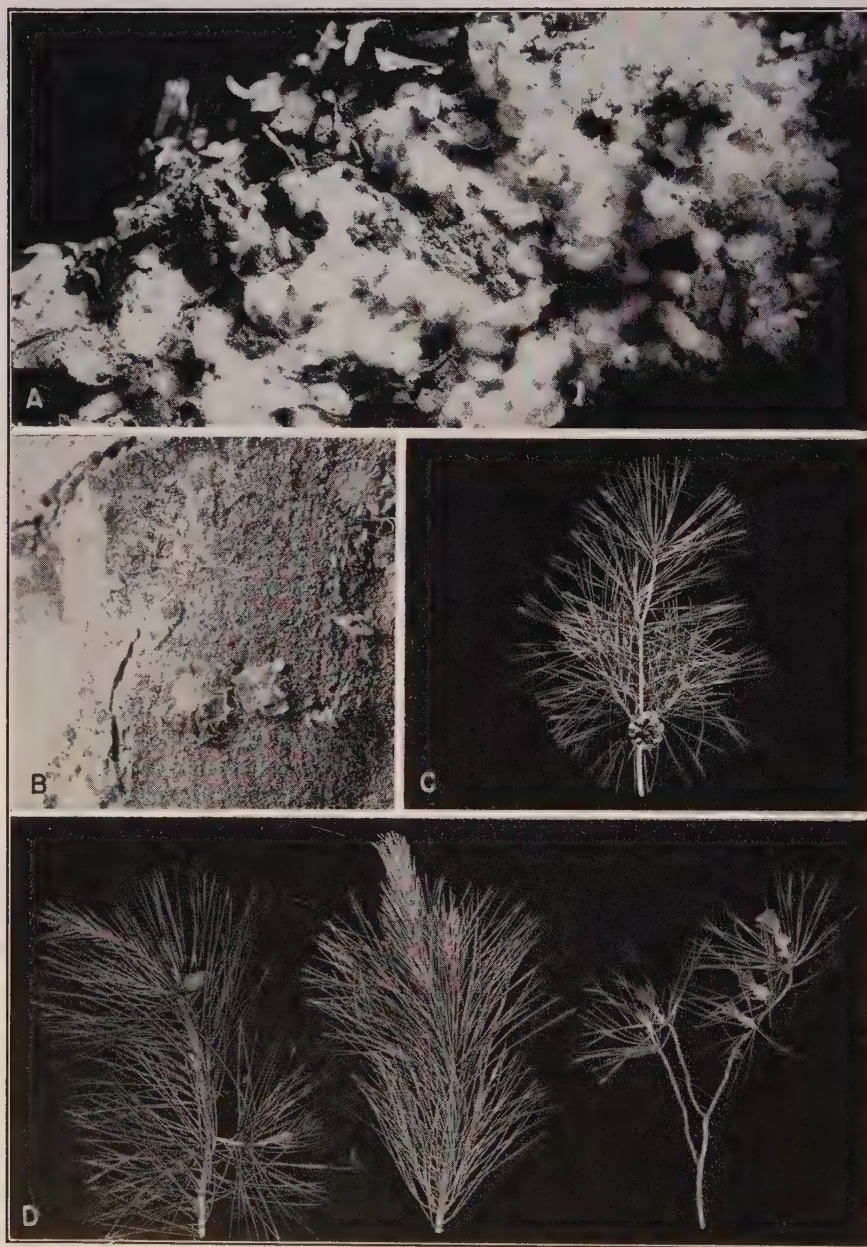


FIGURE 2. Red pine scale (*Matsucoccus resinosa*) and its four hosts. (A) Masses of cocoons on lower side of branch of red pine ($\times 3.7$). (B) Overwintering stage ($\times 6$). (C) *P. densiflora* var. *umbraculifera* ($\times 0.13$). (D) Pine branches, left to right, *Pinus tabulaeformis*, *P. resinosa*, *P. densiflora* ($\times 0.13$).

the months of June, July and August when eggs and crawlers were present. The infested red pine twigs were appressed to the branches throughout the remainder of the growing season. An examination of these trees in the autumn of 1954 for evidence of living scales of this species was negative. However, when the trees were examined on November 9, 1955, a Japanese red pine (*Pinus densiflora* Sieb. & Zucc.) tree which had been exposed as described above on May 26, 1954 and June 5 and August 4, 1955, was found to be infested with living *Matsucoccus* scale (3). Many first-stage nymphs were apparently overwintering on 1951 twig growth. Also several intermediate stages were found on the four- and five-year-old twig growth. The infestation of this tree was equal in density to that of the red pine trees in the infested grove in the arboretum. A Chinese pine (*Pinus tabulaeformis* Carr.) also was found to be infested. This tree likewise had been exposed by having red pine twigs infested with *Matsucoccus resinosae* scale tied to its branches on June 15, and August 4, 1955. Two live larvae were found on the four-year-old twig growth and many exuviae or cast skins were present. No living scales of *Matsucoccus resinosae* were found on any of the other 20 species of pine in the arboretum in 1955.

An examination of the trees on October 30, 1956, showed that *Pinus densiflora* was as heavily infested with *Matsucoccus resinosae* scale as were the red pine trees in the arboretum. A *Pinus densiflora* tree that had not been artificially infested with scale also was found to be infested. The nearest infested tree was 40 ft. distant. In addition four trees of *Pinus densiflora* Sieb. & Zucc. var. *umbraculifera* Mayr. and one tree of *Pinus tabulaeformis* Carr. were lightly infested with living scale. The existence of more than one host of *Matsucoccus resinosae* adds to the complexity in making surveys and in the control of this species. The twig and leaf characteristics of the known hosts of *Matsucoccus resinosae* are depicted in Figure 2 C and D.

No living *Matsucoccus resinosae* scales were found on any of the 19 species of pine in the arboretum as listed below:

Pinus aristata Engelm.

P. armandi Franch.

P. ayacahuite Ehrenb. (*P. hamata* Roetzl.)

P. bungeana Zucc.

P. cembra L. var. *sibirica* Loud.

P. flexilis James

P. griffithii McClelland (*P. excelsa* Wall.)

P. heldreichii Christ. var. *leucodermis* (Ant.) Markgraf ex Fitschen.

P. koraiensis Sieb. & Zucc.

P. lambertiana Dougl.

P. monticola Dougl.

P. mugo Turra var. *rostrata* Hoopes (*P. montana* Mill., *P. uncinata* Ramond.)

- P. nigra poiretiana* (Ant.) Aschers. & Graebn. (*P. laricio* Poir. var. *calabrica* Loud.)
P. parviflora Sieb. & Zucc. (*P. pentaphylla* Mayr.)
P. peuce Griseb.
P. ponderosa Dougl.
P. rigida Mill.
P. sylvestris L. var. *rigensis* Loud.
P. thunbergii Parl.

EFFECTS ON HOSTS

The first visible indication of feeding by this insect is the olive-green color of the current year's foliage on infested trees and the stunted growth. The foliage changes to light yellow and finally to brick red on individual branches and finally over the entire crown. The undersides of these branches show masses of male cocoons resembling small wads of cotton. Death of the tree soon follows. The bark on the branches and the trunk of heavily infested trees has a swollen and cracked appearance. Areas of dead tissue can be found beneath each feeding scale.

NATURAL ENEMIES

A number of predators were observed feeding on *Matsucoccus resinosae* scale on red pine. Among these were two species of ladybird beetles,² *Coccinella transversoguttata* Fald. and *Cleis picta* (Rand.), a dipterous larva and several species of spiders. A small hemipteron, *Xanotrachelliella inimica* D. & H., prefers the eggs and larvae. Attempts to rear insect parasites by caging scale-infested red pine limbs failed. The several species of native predators attacking the scale were never sufficiently abundant to effect any appreciable control.

COLDHARDINESS

EXPERIMENT TO DETERMINE COLDHARDINESS OF OVERWINTERING MATSUCOCCLUS SCALE

Some 40 red pine trees 4 ft. high infested with scale, received March 1, 1954, from Mr. James L. Bean of the U. S. Forest Service, New Haven, Conn., were placed in a 41° F.-cold room at Boyce Thompson Institute until a deep freeze unit was installed in April. The trees which were cut back to 3 feet, and the roots, burlap-wrapped in peat moss, were subjected during the latter part of April and the forepart of May to low temperatures in lots of five. Counts of live scales were made from twig samples (2 in. length) cut from the trees 24 hours before and after treatment. Twig samples were kept at 41° F. until pretreatment and post-treatment counts

² Identified by Mr. John Wilcox, New York State Museum and Science Service, Albany, N. Y.

of live scale were made under a binocular microscope. Following this, three series of pine trees each were run at minimum temperatures of -15° F., -26° F., and -29° F.

On November 23, 1954, some 30 red pine trees 4 ft. high infested with scale were received from Mr. Thomas McIntyre of the U. S. Forest Service, New Haven, Conn. The trees were overwintered in a 41° F. cold room. The trees were prepared for cold treatments as described previously and subjected to temperatures of -10° and -25° F. during the month of May, 1955.

Low temperatures in the 1954 and 1955 series were controlled within one degree Fahrenheit. The results appear in Table I.

It will be seen from Table I that the temperature was lowered at average rates of 0.3° to 3° F. per hour. The duration of the tests ranged from

TABLE I
EFFECT OF TEMPERATURE ON MATSUCOCCLUS SCALE

| Temp., ° F. | Av. rate temp. lowered at beginning of treat- ment, in °F. per hr. | Duration at lowest temp., hrs. | Av. rate temp. raised at end of treat- ment, in °F. per hr. | Av. live scales per 10 sq. in. bark | | % Kill |
|-------------------------|--|--|---|--|-------------------------------|-----------|
| | | | | Pretreat- ment counts | Post-treat- ment counts | |
| First year experiments | | | | | | |
| -15 | 3 | 4 | 5 | 15.8 | 1.0 | 93 |
| -26 | 2 | 4 | 4 | 5.2 | 0.2 | 96 |
| -29 | 1 | 7 | 3 | 13.0 | 0.4 | 97 |
| Second year experiments | | | | | | |
| -25 | 0.3 | 5 | 4 | 11.9 | 1.1 | 91 |
| -25 | 1 | 5 | 1 | 21.4 | 1.2 | 94 |
| -25 | 1 | 4 | 4 | 12.9 | 1.2 | 91 |
| -10 | 1 | 4 | 2 | 12.0 | 1.0 | 92 |
| -10 repeat | 1 | 4 | 3 | — | 0.02 | 99 |
| -10 | 1 | 7 | 2 | 8.6 | 1.9 | 91 |

4 to 7 hours. At the end of the treatments the temperature was raised at average rates of from 1° to 5° F. The total number of insects counted was 1051 and the counts per five trees ranged from about 60 to 150 per sq. in. of bark.

There appeared to be no significant difference between kills obtained with single treatments at any of the temperatures tested. When a treatment at -10° F. was repeated, however, for a period of 7 hours 99 per cent reduction in population was obtained as compared with a kill of 92 per cent for single treatments.

These results indicate that the scale may be sufficiently cold-sensitive

to affect its geographical range northward. It would appear that rapid spread northward would be retarded until a race is evolved that proves to be more resistant to cold than the present race of *Matsucoccus resinosae*.

COLDHARDINESS OF MATSUCOCCUS CRAWLERS

To ascertain the effect of an early frost on the crawler stage of *Matsucoccus resinosae*, two twigs, 3 inches long and $\frac{1}{2}$ inch in diameter heavily infested with crawlers were placed in a large Petri dish, which was then removed to a cold room. The duration of the experiment was 16 hours. The temperature fluctuated between 27° F. and 36° F. The Petri dish, containing the twigs, was removed. An immediate inspection showed that nearly all the crawlers had left the twigs and were at the bottom of the Petri dish.

Two hours after the dish had been removed from the cold room a count was made to determine the ratio of live crawlers to dead. A crawler was considered alive if it moved either antennae or legs. A twig with a total population of 149 crawlers showed a mortality of 64 per cent. A second twig counted three hours later with a total population of 103 crawlers gave a mortality of 53 per cent. These results suggest that the crawler is paralyzed by cold. This response may account for the migration to the base of the Petri dish. The results indicate that a longer exposure or a lower temperature would be required to control the scale.

ACKNOWLEDGMENTS

The writer is especially indebted to Mr. James L. Bean and Mr. Thomas McIntyre of the United States Department of Agriculture Forest Service, New Haven, Connecticut, to Dr. Donald L. Collins and Mr. Donald P. Connola of the State Entomologist's Office, New York State Museum and Science Service, Albany, New York, and to Mr. William E. Smith of the New York State Department of Conservation, Albany, New York, for their cooperation and assistance in identification of *Matsucoccus resinosae* on its hosts.

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EFFECTS OF CHLORINE SUBSTITUTION AND ISOMERISM ON THE INTERACTIONS OF *s*-TRIAZINE DERIVATIVES WITH CONIDIA OF *NEUROSPORA SITOPHILA*

H. P. BURCHFIELD AND ELEANOR E. STORRS

SUMMARY

The uptake of 2,4-dichloro-6-anilino-*s*-triazine and derivatives by conidia of *Neurospora sitophila* was investigated to determine mode of action and establish the relationship between physico-chemical properties and biological activity for this group. These compounds were accumulated very rapidly from dilute aqueous solution by fungus spores. Uptake reached a maximum in from 10 to 50 minutes, after which part of the material was released back into the solution. The possibility that this was caused by changes in the permeabilities of the cell membranes was supported by the finding that intracellular phosphorus was released on similar treatment. When an excess of triazine was present in the aqueous phase, the period of release was followed by another period of uptake at a much slower rate indicating that two mechanisms exist for uptake. However, spores treated in this way were still able to germinate. The amount of material required to reduce germination by 50 per cent was found to be about 1,500 μ g. per gram of spores, or about three to six times the amount required to obtain the release of triazine and radioactive phosphorus.

A considerable quantity of the material which was taken up evidently combined very rapidly with protoplasmic constituents since only 20 to 25 per cent of it could be recovered on immediate extraction and analysis. Thus the biological activity of these compounds probably depends on rapid accumulation, and the ability of the active halogen atoms on the triazine ring to react with sulfhydryl and amino groups in essential metabolites. Systems dependent on compounds other than cysteine and glutathione must be involved since inhibition was only partly reversed when these materials were used as antidotes.

The fungitoxicity of the *s*-triazine to conidia of *N. sitophila* was increased by substituting a chlorine atom in the benzene ring in the order: *p*-chloro > *m*-chloro > *o*-chloro > unsubstituted, but their chemical reactivities with substrates such as cysteine and glycine were found to increase in the order: *o*-chloro > *m*-chloro > *p*-chloro > unsubstituted. However, these compounds were accumulated by spores in amounts proportional to their apparent toxicities. Thus the *p*-chloro isomer was more effective against *N. sitophila* than the *o*-chloro isomer, but on the other hand more of it was accumulated by spores under identical conditions. When toxicity was measured in terms of the amount taken up per gram of spores, the two compounds were equivalent to within 10 per cent.

INTRODUCTION

In previous publications (2, 4) it was shown that 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine is able to combine with metabolic intermediates containing amino and sulfhydryl groups through metathetical reactions involving the active halogen atoms of the heterocyclic ring. However,

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chlorine atoms substituted on the benzene ring must also regulate bioactivity since Schuldt and Wolf (16) have found that 2,4-dichloro-6-anilino-*s*-triazine is less effective for the control of fungus diseases of higher plants than derivatives with a halogen atom in the *ortho*, *meta*, or *para* positions of the benzene ring. Since the latter halogens are very inert chemically, they must enhance fungitoxicity through increasing the amount of chemical which can be taken up by spores, changing the reactivities of the heterocyclic halogen atoms by inductive effects, or possibly through changes in molecular geometry which enable them to compete more successfully with some essential metabolite for a site on an enzyme surface. Compounds closely resembling the *s*-triazines have not been found in nature, and some measure of fungitoxicity is maintained even with major changes in the substituent in the 6-position of the triazine ring (16), so the possibility of their being metabolic antagonists is rather remote. Accordingly the uptake of four triazine derivatives by conidia of *Neurospora sitophila* and their relative reaction rates with cysteine and glycine were determined to establish which of the other two factors predominates in regulating fungitoxicity.

MATERIALS AND METHODS

Chemicals. Samples of 2,4-dichloro-6-anilino-*s*-triazine, 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine, 2,4-dichloro-6-(*m*-chloroanilino)-*s*-triazine, and 2,4-dichloro-6-(*p*-chloroanilino)-*s*-triazine prepared by the method described by Schuldt and Wolf (16) were dissolved in hot benzene and filtered to remove insoluble impurities. The solutions were then decolorized with Norite and the compounds recrystallized from benzene until the melting points were constant (Table I). Stock solutions containing 500 μ g. per ml. were prepared in acetone so that on dilution of 1 ml. with 99 ml. of water an aqueous solution containing 5 μ g. per ml. was obtained. Preparation of the reagents used for analysis has been described elsewhere (3).

Fungus cultures. A conidial strain of *Neurospora sitophila* (Mont.) Shear & Dodge was cultured in wide-mouthed bottles of 200-ml. capacity (8) on the medium recommended by Horowitz (6) for mutants of *Neurospora*

TABLE I
SOLUBILITIES AND MELTING POINTS OF THE *s*-TRIAZINE DERIVATIVES

| Substituent on benzene ring | Melting point, °C. | Solubility* μ g./ml. |
|-----------------------------|-----------------------|-----------------------------|
| Unsub. | 137.5 | 100 |
| <i>o</i> -Chloro | 157.5 | 10 |
| <i>m</i> -Chloro | 131.8 | — |
| <i>p</i> -Chloro | 187.5 | 6.5 |

* In water containing 1 per cent acetone.

crassa except that the DL-methionine was omitted and 30 g. of agar were used per liter as recommended by Miller, McCallan, and Weed (10). Spores were harvested three days after inoculation by the vacuum technique described by McCallan and Wilcoxon (8). The spores were filtered through cheesecloth and centrifuged three or four times from distilled water. After suitable dilution, spore concentrations were determined by counting the conidia in a Fuchs-Rosenthal cell, and calculating the weight of spores in suspension according to the method of Miller, McCallan, and Weed (10) who estimated that one million conidia of *N. sitophila* would weigh 0.347 mg. based on density and size measurements. The spores were then diluted to the concentration desired for each individual experiment.

Measurement of uptake and germination. For measurement of the cumulative uptake of triazines by spores a suspension containing the desired weight of spores was added to a 15-ml. centrifuge tube and spun at *ca.* 1,500 r.p.m. for 5 minutes. The supernatant was decanted off and 10 ml. of triazine solution, usually containing 5 μ g. per ml., added. The spores were immediately resuspended and transferred to 50-ml. flasks. The flasks were then stoppered and shaken in a constant temperature bath maintained at 29° C. for the duration of the exposure period. At the end of this time the suspension was transferred back to the original tube and the spores sedimented by centrifugation. The supernatant was then decanted off and a 5-ml. portion analyzed for triazine by the colorimetric procedure previously described (3). The spores were then resuspended in a fresh solution of triazine (10 ml.) and treated as before. This process was repeated until the amount taken up at each treatment became small. The amount taken up on each treatment was calculated as the difference between the amount originally in solution and the amount remaining after exposure to the spores. It was demonstrated that filtrates from spore suspensions do not react with the triazines. The cumulative amount taken up was calculated as the sum of the amounts taken up on each treatment. In all cases where it was desired to compare different treatments, the tests were run at the same time on aliquots from the same suspension of spores.

In measuring the rates of uptake and release of triazines by spores, 1 ml. of the stock solution of triazine in acetone was diluted with 89 ml. of water and the spores were then added in an additional 10 ml. of water to make the total volume 100 ml. The time of mixing was noted, and 20 to 30 seconds later approximately 10 ml. of the suspension were filtered rapidly through a sintered glass funnel of medium porosity under vacuum. The filtrate was collected in a test tube and reserved for analysis. Additional samples were taken at approximately 2, 4, 8, 16, 32, 64, and 128 minutes after mixing and treated in the same manner. The time intervals were not uniform since it was often desired to run a group of experiments at the same time. At the conclusion of each experiment, 5-ml. aliquots of

the filtrates were analyzed for the triazine (3). Since it was found that the sintered glass funnels adsorbed small amounts of triazine, each experiment was run using a single funnel and the spores removed with a rubber policeman between readings. The results were then corrected with reference to a calibration curve made for each individual funnel relating the amount of triazine adsorbed to the total concentration.

ED₅₀ values on a spore weight basis were determined by exposing 50 mg. of spores in a centrifuge tube to 10 ml. of solution containing 50 μ g. of triazine so that the maximum possible uptake was 1000 μ g. per gram. The suspensions were then shaken in a water bath at 29° C. for one hour, centrifuged, and the supernatants reserved for analysis in order to determine the actual dosage. A second aliquot of spores was treated twice in this manner and a third aliquot three times etc. until six groups of spores were obtained which had been treated from one to six times with the triazine. The cumulative amount taken up in all the treatments was calculated for each group of spores in order to obtain the dosage on a spore weight basis. With the compounds which were taken up most readily, the dosage-response curves were so steep that it was necessary to use intermediate treatments in which the spores were exposed to smaller amounts of triazine. The spore suspensions were then diluted to 200,000 per ml., and 1-ml. aliquots were added to 1 ml. of double strength Fries medium (15). Drops of the suspensions were then placed on glass slides and incubated in a moist chamber for 18 hours at 18° C. At the end of this time the percentage of spores ungerminated was determined by the usual procedure (1). The percentage of spores ungerminated was plotted in probit units against the dose, and the μ g. of chemical per gram of spores required to reduce germination by 50 per cent in comparison with an untreated control was estimated by interpolation.

Apparent ED₅₀ values based on the amount of toxicant in solution rather than the amount taken up by the spores were determined by the recommended method of the American Phytopathological Society (1). Fries medium was added to the suspensions to induce germination and readings were made after 18 hours' incubation at 18° C. In order to observe differences in mycelial growth, 4-ml. aliquots of the same preparations were incubated at 25° C. for 48 hours and the mycelial mats transferred to watch glasses and photographed.

The effect of removal of the excess toxicant on spore germination was investigated by exposing spores to concentrations of chemical ranging from 0.2 to 1.8 μ g. per ml. for 24 hours in the absence of Fries medium and then filtering the suspensions through a sintered glass funnel. The spores were resuspended in distilled water at a concentration of 200,000 per ml. and then diluted with an equal volume of double strength Fries medium. The percentage of spores ungerminated after 18 hours at 18° C. was estimated in the usual way.

Reactions with spores. The amount of triazine reacting with spore constituents was determined by permitting 300 mg. of spores to remain in contact with 30 ml. of solutions containing 5 μ g. of triazine per ml. for various lengths of time and then filtering the suspensions through sintered glass funnels. Five-ml. aliquots of the filtrates were then analyzed and the amount of triazine taken up calculated as the difference between the amount originally present and the amount recovered after exposure to the spores. The spores were then transferred to a 50-ml. beaker, extracted with 2 per cent acetyl chloride in acetone, and the amount of triazine recovered determined by the petroleum ether extraction method described elsewhere (3). The amount reacted with the spores was calculated as the difference between the amount taken up by the spores and the amount recovered by this latter treatment.

Oxygen uptake. The effect of triazines on oxygen uptake by conidia of *N. sitophila* was determined by treating 100 mg. of spores with solutions containing 5 μ g. of the triazine per ml. for one hour. The suspensions were filtered through sintered glass funnels and the filtrates analyzed for triazine to determine the amounts taken up. The spores were then resuspended in 10 ml. of water and 1-ml. aliquots added to Barcroft-Warburg vessels. One ml. of *M*/30 phosphate buffer at a pH of 2.93 containing 5 mg. of pyruvic acid was added to each vessel and 0.2 ml. of 10 per cent sodium hydroxide placed in the center wells. A pH of 2.9 was selected since previous experiments had shown this to be the point of maximum oxygen uptake probably because of the preferential assimilation of undissociated pyruvic acid. The vessels were then attached to manometers and readings made at half-hour intervals over a period of five hours. Results were calculated in terms of microliters of oxygen consumed per hour.

Solubilities and reaction rates. The solubilities of the compounds in water containing 1 per cent acetone and their reaction rates with cysteine and glycine were determined at 29° C. and pH 7.0 by the methods described elsewhere (3, 4).

EXPERIMENTAL RESULTS

UPTAKE BY SPORES

Rates of uptake of the triazines by conidia of *N. sitophila* were first investigated using suspensions containing 500 μ g. of chemical per gram of spores. This was very convenient from an analytical standpoint, since the absorbance obtained in the color reaction (3) varied from about 1.0 for the initial reading to about 0.01 after the maximum amount of chemical had been taken up. The experiments were run at a concentration of 5 μ g. of compound per ml. of solution, which insured that all of the compounds would be in true solution in 1 per cent aqueous acetone (Table I). Suspensions containing the triazines and the spores were prepared at room temperature and samples withdrawn at intervals for filtration and analysis as

described previously. In experiments designed to compare differences in the behavior of the four compounds, spores from the same batch at about the same age were used in order to minimize biological variation.

The uptake of all four compounds was very rapid for the first few minutes, but thereafter leveled off to reach a maximum, after which the amount of triazine in the aqueous solution apparently increased (Fig. 1). The compound unsubstituted in the benzene ring and the *o*-chloro derivative were taken up more slowly in the *initial* stages than the *meta* (Fig. 2)

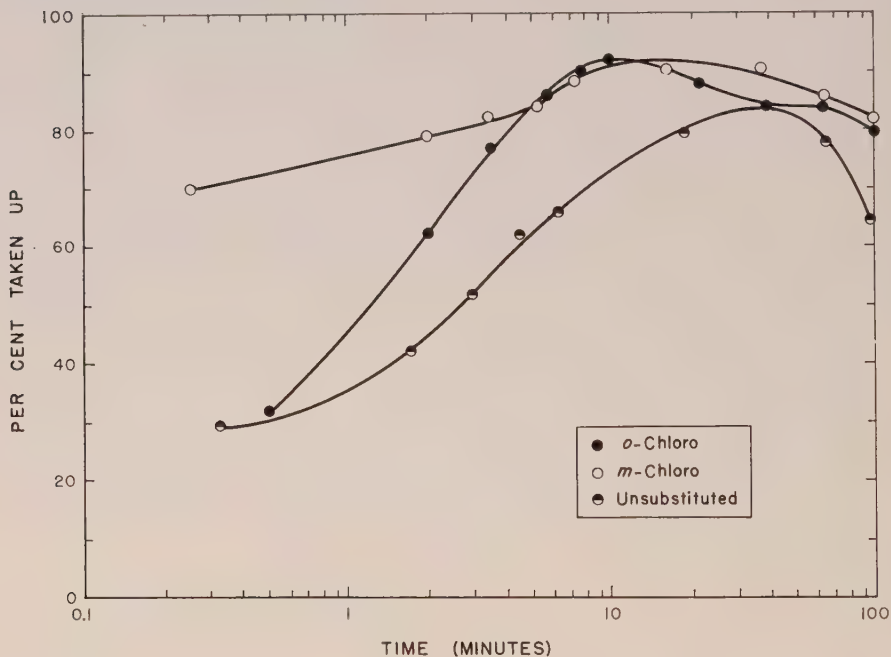


FIGURE 1. Effects of halogen substitution in the *ortho* and *meta* positions on the uptake and release of 2,4-dichloro-6-anilino-*s*-triazine by conidia of *N. sitophila* when exposed to solutions containing 500 μ g. of compound per gram of spores.

or *para* derivatives but in all cases over 80 per cent of the available material was removed from the aqueous solution at maximum uptake. The time required to reach the maximum was about 45 minutes for the unsubstituted compound and about 10 minutes for the *o*-chloro derivative, while the times for the *meta* and *para* derivatives were intermediate. From these data (Table II) it appears that the *meta* and *para* isomers were taken up more rapidly during the first minute than the other two compounds, although the *ortho* compound was more effective in producing physiological changes in the spores which led to the release of part of the material taken

TABLE II

UPTAKE AND RELEASE OF TRIAZINE DERIVATIVES BY CONIDIA OF NEUROSPORA SITOPHILA WHEN EXPOSED TO SOLUTIONS CONTAINING 500 μ g. OF CHEMICAL PER GRAM OF SPORES

| Quantity measured | Derivative | | | |
|-------------------------------------|------------|------------------|------------------|------------------|
| | Unsub. | <i>o</i> -Chloro | <i>m</i> -Chloro | <i>p</i> -Chloro |
| Per cent taken up in first minute* | 35 | 45 | 75 | 70 |
| Per cent taken up at maximum* | 85 | 90 | 90 | 95 |
| Minutes required for maximum uptake | 45 | 10 | 30 | 30 |
| Per cent released at 100 minutes* | 25 | 15 | 15 | 5 |

* Per cent of total available in external solution.

up. The unsubstituted compound was removed from solution least readily and required the longest time to reach maximum uptake.

The effect of spore concentration on uptake was studied by treating 25, 50, 100, and 200 mg. of spores with 50 μ g. of the *o*-chloro derivative in 50 ml. of solution and withdrawing samples for analysis at various time intervals. When the weight of spores was high in proportion to the amount of chemical, uptake was very rapid and quickly reached a maximum, after

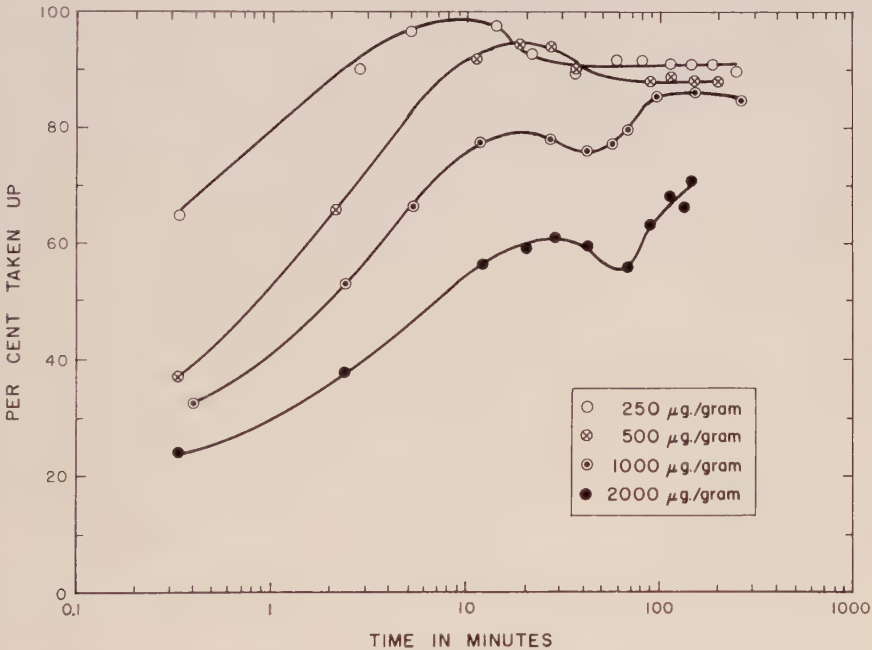


FIGURE 2. Uptake and release of the *o*-chloro triazine derivative at four concentrations of spores and 5 μ g. per ml. of triazine with the total amount of chemical available expressed as μ g. per gram of spores.

which part of the compound was released back into the solution as determined previously. However, when the spore concentration was reduced so that the total available triazine was 1,000 and 2,000 $\mu\text{g.}$ per gram, curves of a different type were obtained (Fig. 2). Initial uptake was much slower, and only 80 and 60 per cent respectively of the triazine was taken up at the maximum. Material was then apparently released until a minimum was reached, after which the net uptake again increased. This effect was observed only when the concentration of chemical with respect to the spores was high, so evidently two mechanisms exist for uptake. One of them was observed in dilute solution and the other only when the concentration of triazine remaining in the external environment at maximum uptake was

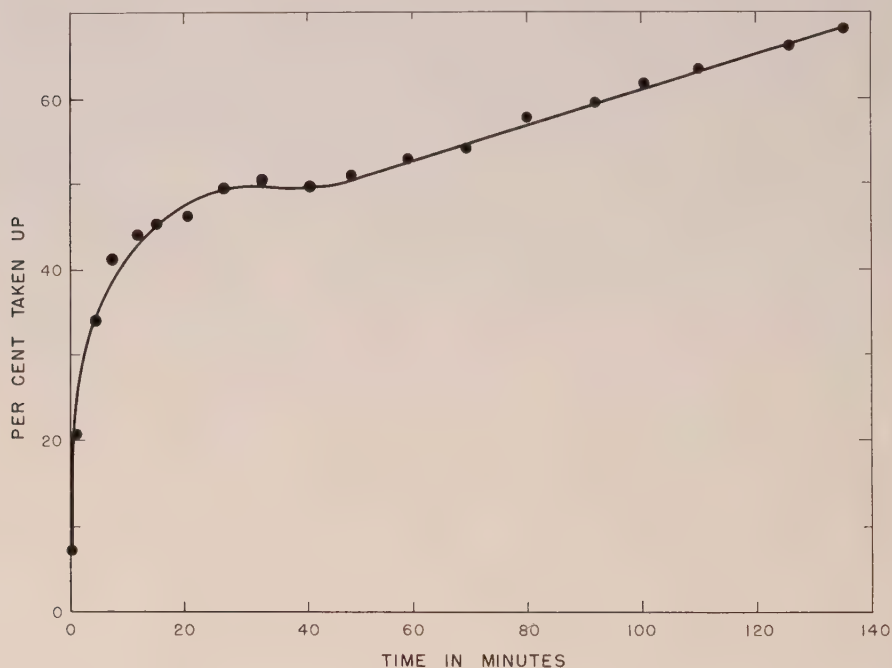


FIGURE 3. Uptake of the *o*-chloro triazine derivative by conidia of *N. sitophila* in amounts equivalent to 2,000 $\mu\text{g.}$ per gram of spores showing lag period in the uptake rate without reversal.

high. In some cases definite maxima and minima were not obtained, but it was always possible to show the existence of a lag period in the uptake curve (Fig. 3), indicating a region where net uptake of the chemical slowed down or ceased and was then resumed.

Since the observed differences in concentration were often rather small, acceptance of the hypothesis that materials taken up by spores can again

be released would be questionable if supported by only a few observations. However, the simplicity of the analytical method made it possible to obtain from 10 to 20 points for each curve, and when it is taken into account that 16 independent experiments were made in which net uptake ceased or was reversed after the initial period of accumulation, there can be little doubt that this phenomenon actually occurred.

Further evidence that the triazines are released by spores was obtained by treating 100 mg. of conidia with 50 μ g. of the *o*-chloro derivative for 20 minutes and filtering off and analyzing the supernatant. The spores were then washed with 2 ml. of water and resuspended in 100 ml. of distilled water. Analyses of aliquots from this suspension made at different time intervals demonstrated a gradual accumulation of triazine in the aqueous phase, so that after 90 minutes 34 μ g. or 7.5 per cent of the ma-

TABLE III

CUMULATIVE RELEASE OF RADIOACTIVE PHOSPHORUS FROM CONIDIA OF *NEUROSPORA SITOPIHILA* BY 2,4-DICHLORO-6-(*o*-CHLOROANILINO)-*s*-TRIAZINE AT VARIOUS TIME INTERVALS AFTER TREATMENT

| Amount of compound available (μ g. per gram) | Cumulative percentage of phosphorus released after various time intervals in minutes | | | | | |
|--|--|----|----|----|----|-------|
| | 5 | 10 | 20 | 40 | 80 | 1,200 |
| 0 | 5 | 2 | 2 | 2 | 3 | 4 |
| 250 | 2 | 2 | 7 | 3 | 9 | 4 |
| 500 | 1 | 3 | 11 | 11 | 8 | 7 |
| 1,000 | 3 | 11 | 16 | 19 | 20 | 22 |

terial taken up was released into the solution. Evidently the accumulation of these materials is a reversible process, and the compounds tend to diffuse from the spores into the medium when the external concentration is low.

Since Miller and McCallan (9) have shown that silver causes the release of radioactive phosphorus from spores grown on a labeled medium, the effect of 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine on phosphorus release was investigated. Labeled conidia were treated with three doses of the triazine for various time intervals, the spores removed by centrifugation, and the supernatants tested for radioactivity. It was found that a total available dose of 500 μ g. of triazine per gram of spores caused a significant release of phosphorus within 20 minutes, while at 1,000 μ g. per gram 22 per cent of the total phosphorus was released at the longest exposure period compared to 4 per cent for the control (Table III). Thus it was possible to demonstrate that the triazines produce changes in the cell membranes which permit the outward movement of protoplasmic constituents.

Although these compounds appeared to have a pronounced effect on the permeability of the spore membranes at doses ranging from 245 to

790 $\mu\text{g.}$ per gram, the percentage of spores germinated after 20 hours' incubation in Fries medium was found to be equivalent to the controls. Furthermore, oxygen uptake measurements made on spores treated with the four compounds in this range failed to show any marked differences in their ability to utilize pyruvate (Table IV). It became apparent that much larger amounts of these materials would have to be taken up in order to interfere with metabolic processes, so a study was made of their accumulation by spores on consecutive treatments.

Experiments made with the *o*-chloro compound at a concentration of 500 $\mu\text{g.}$ of chemical per gram of spores showed that 95 per cent of the available material was taken up during the first treatment, but only 65 per cent during the second. On each subsequent treatment the amount taken

TABLE IV
EFFECT OF TRIAZINE DERIVATIVES ON OXYGEN UPTAKE BY 100 MG. OF CONIDIA
OF NEUROSPORA SITOPHILA ON A PYRUVATE SUBSTRATE
BUFFERED AT A PH OF 2.9

| Derivative | Dosage ($\mu\text{g.}$ per gram) | Microliters O_2 per hour | Per cent of control |
|------------------|--------------------------------------|--------------------------------------|------------------------|
| Unsub. | 430 | 60 | 80 |
| <i>o</i> -Chloro | 450 | 65 | 90 |
| <i>m</i> -Chloro | 460 | 70 | 100 |
| <i>p</i> -Chloro | 470 | 80 | 115 |
| Control | — | 70 | — |

up was less except when the time of exposure was considerably longer. After five consecutive treatments extending over a period of 20 hours the cumulative amount taken up was 1,400 $\mu\text{g.}$ per gram of spores and germination was still only slightly impaired.

The four compounds were then compared in the same experiment to determine the effects of chlorine substitution and isomerism on affinity for spores. It was found that the substitution of a single chlorine atom in the *ortho* position of the benzene ring increased total uptake by 30 per cent over the unsubstituted compound, while substitution in the *meta* or *para* positions increased it by 80 per cent (Table V). The difference of 30 $\mu\text{g.}$ in uptake between the latter compounds is not regarded as significant. Thus the affinity of the toxicants for the spores was found to be in the order *p*-chloro = *m*-chloro > *o*-chloro > unsub. in these experiments.

However, it was considered possible that this order of affinity might be modified under the conditions of the slide-germination test (1) where a much larger amount of chemical is used in comparison to the weight of the spores. In this test only 10^5 spores suspended in 2 ml. of medium are used, so when the external concentration is 1 $\mu\text{g.}$ of toxicant per ml. of solution, the total available dose would be 5.8×10^4 $\mu\text{g.}$ per gram of spores. The

TABLE V

COMPARATIVE UPTAKE OF TRIAZINE DERIVATIVES BY CONIDIA OF NEUROSPORA SITOPHILA IN CONSECUTIVE TREATMENTS WITH 500 μ G. OF CHEMICAL PER GRAM OF SPORES

| Exposure time (hours) | Amount taken up by spores (μ g. per gram) | | | |
|-----------------------|--|------------------|------------------|------------------|
| | Unsub. | <i>o</i> -Chloro | <i>m</i> -Chloro | <i>p</i> -Chloro |
| 0.5 | 440 | 480 | 480 | 480 |
| 1.5 | 250 | 320 | 410 | 430 |
| 1.3 | 110 | 170 | 340 | 340 |
| 1.0 | 80 | 140 | 290 | 300 |
| 16 | 210 | 290 | 400 | 400 |
| Total | 1,090 | 1,400 | 1,920 | 1,950 |

amount of triazine that would be taken up under these conditions would be very small in proportion to the amount present, and it would not be possible to make a satisfactory analytical measurement of the difference. However, it was possible to approximate this condition by holding the amount of triazine constant and progressively reducing the weight of spores until a point was reached where the difference between the amount of toxicant initially present and the amount present after exposure to the spores was small but still accurately measurable. It was found that as the weight of spores was reduced, the amount taken up on a spore weight basis during two hours' exposure became progressively larger, but not in proportion to the total material available (Table VI). Below 16 mg. of spores, additional uptake was negligible and in some cases reversals were obtained. Since this more nearly approximates the conditions that would occur in a slide-germination test, the limiting values for relative uptake at 16 mg. of spores should provide a better measure of the affinity of the various chemicals for the spores than the uptake obtained on consecutive treatments.

Under these conditions the order of uptake was *p*-chloro > *m*-chloro > *o*-chloro > unsub. which agreed with that previously found except that the *para* isomer was accumulated in larger amounts than the *meta* isomer

TABLE VI

UPTAKE OF TRIAZINE DERIVATIVES BY DIFFERENT AMOUNTS OF CONIDIA OF NEUROSPORA SITOPHILA DURING TWO HOURS' EXPOSURE TO SOLUTIONS CONTAINING 2.5 μ G. OF COMPOUND PER ML.

| Weight of spores (mg.) | Maximum possible dose (μ g. per gram) | Uptake of compound (μ g. per gram) | | | |
|------------------------|--|---|------------------|------------------|------------------|
| | | Unsub. | <i>o</i> -Chloro | <i>m</i> -Chloro | <i>p</i> -Chloro |
| 250 | 100 | 90 | 90 | 95 | 95 |
| 125 | 200 | 140 | 160 | 180 | 180 |
| 63 | 400 | 170 | 200 | 290 | 300 |
| 31 | 800 | 190 | 240 | 400 | 430 |
| 16 | 1,600 | 200 | 270 | 400 | 510 |

The sites at which these compounds are accumulated by the spores appear to be the same. In experiments in which conidia were treated first with one compound and then another, the amount accumulated in the second treatment was influenced by the first. For example, spores exposed first to the triazine unsubstituted in the benzene ring and then to the *o*-chloro derivative showed reduced uptake in the second treatment (Table VII) similar to that obtained when the spores were subjected to two consecutive treatments with the same compound. Reversing the order of treat-

TABLE VII

UPTAKE OF (A) 2,4-DICHLORO-6-ANILINO-5-TRIAZINE, and (B) 2,4-DICHLORO-6-(*o*-CHLORO-ANILINO)-5-TRIAZINE IN CONSECUTIVE MIXED TREATMENTS FROM SUSPENSIONS CONTAINING 1,000 μ G. OF CHEMICAL PER GRAM OF CONIDIA OF *NEUROSPORA SITOPHILA*

| Order of treatment | Initial uptake (μ g. per gram) | Second treatment (μ g. per gram) | Total uptake (μ g. per gram) | Per cent of available material taken up |
|--------------------|-------------------------------------|---------------------------------------|-----------------------------------|---|
| A then A | 530 | 200 | 730 | 37 |
| B then B | 600 | 230 | 830 | 42 |
| A then B | 540 | 270 | 810 | 41 |
| B then A | 630 | 200 | 830 | 42 |

ment had no effect, since prior treatment with the *o*-chloro derivative reduced the amount of unsubstituted compound that could be taken up. If different sites of action were involved it would be expected that the two compounds would be taken up independently (11). It appears, therefore, that these compounds are accumulated at the same receptor sites but probably in different amounts.

INHIBITION OF GERMINATION

Once the general pattern of uptake of these compounds by fungus spores was established, it became of interest to determine how they would affect germination and whether this could be correlated with the amount of each material the spores were capable of accumulating. After preliminary investigations to determine the range of activity, spore germination tests were run at concentrations of 0.2 to 1.8 μ g. per ml. of the four compounds. The results showed that the fungitoxicity of these compounds to conidia of *N. sitophila* increased in the same order that was observed for their accumulation from dilute aqueous solution (Table VIII). Substitution of a halogen atom in the benzene ring increased apparent toxicity in all three cases, but *para* and *meta* substitutions were more effective than when the chlorine was placed in the *ortho* position. Thus it appears likely that the order of toxicity of these compounds is dependent primarily upon the amounts that can be accumulated by the spores. Inhibition of mycelial growth after an additional 24 hours' incubation at room temperature

(Fig. 4) indicated the same general order of effectiveness and confirmed the results of the glass slide-germination tests.

These experiments serve to show that the triazines are effective fungistats, which can inhibit the germination of spores when present in the medium. However, the large amounts of these compounds which were accumulated by spores in the studies on uptake without inhibiting germination, made it of interest to determine whether they would have fungicidal properties when the spores were first treated with chemical and then transferred to a culture medium. Spores were exposed to solutions of the tria-



FIGURE 4. Mycelial growth of *N. sitophila* in the presence of various concentrations of triazine derivatives expressed as $\mu\text{g. per ml.}$ of solution: (A) *p*-chloro, (B) *m*-chloro, (C) *o*-chloro, (D) unsubstituted.

zines containing 0.2 to 1.8 $\mu\text{g. per ml.}$ in the absence of biotin (5) and were then filtered, washed, and resuspended in water. Eighteen hours later Fries medium was added and germination counts were made after an additional 18 hours' incubation. Inhibition of germination by the *p*- and *m*-chloro derivatives was approximately the same as obtained in the previous test (Table VIII), but the *o*-chloro derivative and the unsubstituted compound were considerably less effective. Evidently these latter two compounds were not taken up in sufficiently large amounts during the initial incubation period to maintain good control of germination after removal from the solutions of toxicants. Thus the *p*- and *m*-chloro derivatives were effective both as fungicides and fungistats, while the *ortho* isomer and the unsubstituted compound had comparatively weak fungicidal properties.

In view of the inverse relationship between relative uptake and spore germination obtained on these compounds, investigations were next made to determine the amount of material on a spore weight basis required to inhibit germination of 50 per cent of the spores in order to confirm that differences in apparent toxicity were caused primarily by differences in the amounts assimilable. Since the materials were taken up very slowly from solution in comparison to their intrinsic toxicities it was necessary to resort to successive treatments to build up lethal doses within a reasonable length of time. A suitable dosage range for the *ortho* isomer was obtained with three to six exposures of one hour each to solutions containing 5 μ g. per ml. applied at a rate of 1,000 μ g. of chemical per gram of spores. The *para* and *meta* isomers required two to five similar treatments except that it was necessary to interpose five shorter treatments between the second and

TABLE VIII

EFFECTIVENESS OF TRIAZINE DERIVATIVES IN PREVENTING GERMINATION OF CONIDIA OF NEUROSPORA SITOPHILA IN COMPARISON TO RELATIVE UPTAKE FROM DILUTE SOLUTION

| Derivative | ED ₅₀ (μ g. per ml.) | | Relative uptake by spores |
|------------------|--------------------------------------|-----------------|---------------------------|
| | Fungistatic test | Fungitoxic test | |
| <i>p</i> -Chloro | 0.33 | 0.3 | 2.5 |
| <i>m</i> -Chloro | 0.44 | 0.4 | 2.0 |
| <i>o</i> -Chloro | 0.56 | 1.0 | 1.3 |
| Unsub. | 0.62 | >1.8 | 1.0 |

third full exposures to secure doses within the required range. These were obtained by allowing spores to remain in contact with the solutions for 6, 12, 18, 30, and 45 minutes after the second one-hour exposure. It was not possible to build up a high enough concentration of the unsubstituted compound within the spores to prevent germination by any of the procedures investigated. Because of the large number of analyses required to estimate cumulative uptake on successive treatments, all three active materials could not be included in the same experiment. However, when the compounds were tested in pairs in successive experiments, it was found that they had the same order of toxicity. In order to establish this more precisely, the *ortho* and *para* isomers were compared in six independent experiments with different lots of spores. Average ED₅₀ values were 1,530 and 1,670 μ g. per gram respectively. This difference was found to be significant at the 5 per cent level but not at the 1 per cent level. Thus the probability is fairly good that the *ortho* isomer is slightly more toxic than the *para* compound on a spore weight basis, but the difference is small in comparison to the difference in uptake (Table VIII).

REVERSAL OF INHIBITION

Since reactions with sulfhydryl compounds had been considered one of the mechanisms by which the triazines might inhibit the germination of spores, reversal of inhibition by the addition of glutathione and cysteine to the medium was investigated. These compounds were known to react with the triazines quite readily in dilute aqueous solution, so it was recognized that adding them to a suspension of spores containing the chemical under the conditions of the slide-germination test would not give a true measure of reversal since they could function by reacting with the triazines before large enough amounts of the latter compounds had been accumulated to prevent germination. In view of this, studies on the reversal of inhibition were carried out by subjecting the spores to successive treat-

TABLE IX

EFFECTS OF GLUTATHIONE, CYSTEINE, AND INCUBATION ON GERMINATION OF CONIDIA OF *NEUROSPORA SITOPHILA* CONTAINING VARIOUS AMOUNTS OF 2,4-DICHLORO-6-(*p*-CHLOROANILINO)-*s*-TRIAZINE

| Amount of triazine taken up (μ g. per gram) | Per cent ungerminated | | | | | |
|--|-----------------------|----------------------|------------------------------|----------------------|---------------------------|----------------------|
| | In Fries medium | | In Fries medium +glutathione | | In Fries medium +cysteine | |
| | Added immediately | Added after 24 hours | Added immediately | Added after 24 hours | Added immediately | Added after 24 hours |
| 2,540 | 100 | 100 | 100 | 100 | 100 | 100 |
| 2,330 | 100 | 100 | 99 | 99 | 100 | 100 |
| 2,160 | 99 | 93 | 95 | 91 | 99 | 91 |
| 1,860 | 86 | 47 | 74 | 31 | 77 | 33 |
| 1,840 | 82 | 31 | 56 | 21 | 68 | 28 |
| 1,750 | 65 | 19 | 27 | 17 | 49 | 22 |
| 1,720 | 38 | 14 | 17 | 14 | 31 | 16 |
| 1,650 | 21 | 16 | 12 | 8 | 23 | 14 |

ments with the triazine, and adding the sulfhydryl compounds to the media containing the spores after the unaccumulated triazine had been removed by filtration. Results obtained with the *para* isomer indicated that neither cysteine nor glutathione was capable of reversing inhibition when the dosage was above 2,300 μ g. per gram of spores and 100 per cent of the spores in the control experiment were ungerminated (Table IX). However, at intermediate doses, where inhibition in the controls was incomplete, partial reversals were obtained with both glutathione and cysteine. The largest effect was observed at a dose of 1,750 μ g. per gram where 65 per cent of the spores in the control failed to germinate compared to 49 per cent in the presence of cysteine and 27 per cent in the presence of glutathione. Since 400 spores were counted in each test these results are highly

significant. Evidently glutathione, and cysteine, were able to reverse inhibition partially if the initial dose was not too high.

A similar effect was obtained by incubating the spores overnight in the absence of cysteine or glutathione before stimulating germination by the addition of biotin. At a dose of 1,750 μ g. of triazine per gram of spores, the percentage of spores ungerminated was reduced from 70 to 20 per cent. Apparently incubation was just as effective in reversing inhibition as the immediate addition of glutathione.

Evidently glutathione and cysteine were able to reduce inhibition either by detoxifying unreacted triazine or by increasing the sulfhydryl level in the spores. Incubation had a similar effect, indicating that the spores were able to repair in part the damage caused by the triazines if the initial dose was not too high. These observations suggest that the primary reactions of the triazines within the spores might be with glutathione and other sulfhydryl compounds. However, exposure to high doses produced permanent injury, probably through secondary reactions with systems which could not be regenerated.

REACTION WITH SPORE CONSTITUENTS

Since the method developed for the analysis of the triazines was found to be semi-specific for the unreacted compound, some preliminary investigations were made to determine how much of the material taken up by the spores is utilized in reactions with cellular constituents. It was assumed that the difference between the amount of triazine taken up from aqueous solution and the triazine recoverable by extraction would represent the amount fixed by the spores since the colors produced by intermediate reaction products containing only one labile halogen atom have much lower intensities (4). Up to the present, work has been confined to the *o*-chloro derivative, but eventually other isomers will be investigated with particular reference to their reactions with specific metabolic intermediates.

Before analyzing for residual triazine it was first necessary to develop a method for extracting it from spores. Because of the non-polar nature of the compound it was believed that it would be effectively removed by any system capable of extracting the orange carotenoid pigments found in *Neurospora*. Alcohol, ether, and benzene were ineffective, but acetone extracted a considerable amount of the pigment. It was found that the incorporation of 2 per cent acetyl chloride in the acetone caused collapse of the spores and resulted in the recovery of most of the pigment in a single extraction. However, the pigment interfered with the colorimetric measurement of triazine so it was necessary to use a modified procedure in which the reaction was carried out in anhydrous pyridine (3) and the product partitioned between petroleum ether and water. The use of acetyl chloride in the extraction medium was necessary for the recovery of triazine, for

when spores containing about 400 $\mu\text{g.}$ per gram were extracted with acetone alone none of the material was recovered while in the presence of acetyl chloride recoveries ranged from 15 to 35 per cent. The amounts recovered could not be increased further either by successive extractions or by increasing the amount of acetyl chloride to 10 per cent.

In addition to facilitating extraction, the acetyl chloride also served to prevent reaction of the triazines with compounds liberated during the extraction process by combining competitively with amino and sulfhydryl groups as was observed to be the case with extracts of green plants. In its absence, the disappearance of triazine could be caused by reactions with compounds liberated during extraction as well as reactions within intact spores. The fact that none of the triazine could be recovered in the absence of acetyl chloride suggests that this was probably the case, for when triazine and spores were added simultaneously to a solution of acetyl chloride only 5 to 15 per cent could not be accounted for by analysis.

TABLE X

RECOVERY OF 2,4-DICHLORO-6-(*o*-CHLOROANILINO)-5-TRIAZINE FROM CONIDIA OF NEUROSPORA SITOPHILA AFTER EXPOSURE TO SOLUTIONS CONTAINING 1,000 $\mu\text{G.}$ OF COMPOUND PER GRAM OF SPORES

| Exposure time (minutes) | Amount taken up ($\mu\text{g.}$ per gram) | Amount recovered ($\mu\text{g.}$ per gram) | Per cent reacted |
|-------------------------|--|---|------------------|
| 1 | 320 | 80 | 75 |
| 7 | 530 | 190 | 64 |
| 20 | 530 | 110 | 79 |

The observation that only 15 to 35 per cent of the triazine taken up by spores could be recovered by extraction indicated that considerable amounts must have reacted with cellular constituents (Table X), and that the greater part of this reaction must have taken place before disruption of the spores by the solvent. In a series of seven experiments the average amount of triazine that could not be accounted for was 380 $\mu\text{g.}$ per gram. Even assuming that 10 per cent was lost during extraction this would correspond to the immobilization of 1.25 micromols of metabolic intermediates such as cysteine or glutathione per gram of spores. Apparently this takes place very rapidly, since in an experiment in which spores were exposed to a triazine solution for only one minute and then immediately extracted, 240 $\mu\text{g.}$ of the material could not be accounted for. At an exposure time of 20 minutes the amount taken up varied from 480 to 590 $\mu\text{g.}$ per gram while the amount recovered varied between 80 and 160 $\mu\text{g.}$ per gram on different batches of spores. However, this does not necessarily mean that uptake by spores is less variable than their potential for reaction since complicating factors may be present in the extraction procedure. In

any case it seems safe to conclude that spores of *N. sitophila* are capable of immobilizing from 240 to 480 $\mu\text{g.}$ of triazine per gram very rapidly after uptake.

Apparently slower reactions can also take place. This was demonstrated in experiments in which spores were exposed to various concentrations of triazine for 20 minutes and a portion of them extracted and analyzed immediately. The remaining spores were then transferred to 10 ml. of distilled water and analyzed after one hour. The results (Table XI) showed that from 10 to 60 $\mu\text{g.}$ of additional triazine disappeared during that time, depending upon the initial dose. These slow reactions may be more important in interrupting metabolic processes than the initial rapid ones since approximately 1,500 $\mu\text{g.}$ per gram must be taken up in order to reduce germination by 50 per cent.

TABLE XI

COMPARISON OF AMOUNTS OF 2,4-DICHLORO-6-(*o*-CHLOROANILINO)-5-TRIAZINE RECOVERABLE FROM SPORES OF *NEUROSPORA SITOPHILA* ON EXTRACTING IMMEDIATELY AND AFTER HOLDING FOR ONE HOUR

| Dosage ($\mu\text{g.}$ per gram) | | Amount extractable ($\mu\text{g.}$ per gram) | | Reacted during one hour ($\mu\text{g.}$ per gram) |
|-----------------------------------|----------|---|----------------|--|
| Available | Taken up | Immediately | After one hour | |
| 125 | 120 | 10 | 0 | 10 |
| 250 | 240 | 50 | 10 | 40 |
| 1,000 | 450 | 140 | 80 | 60 |

RELATIVE REACTIVITIES

After establishing the relationship between chemical structure and uptake by spores for this series of compounds their relative reaction rates with typical metabolic intermediates were measured to determine if these values were also modified by halogen substitution.

Preliminary to this, measurements of the hydrolysis rates of the triazines were made in *M*/30 phosphate buffer at pH 7.0 to find out whether the compounds were stable in the environment, and to determine whether this reaction would be competitive with reactions with amino acids and peptides in dilute aqueous solution. The half life of the *o*-chloro derivative was found to be over 20 days while the other compounds were more stable. Since the half lives in the presence of the substrates that were investigated ranged from 50 to 200 minutes, competition for the triazines from this source would be negligibly small.

The relative reactivities of the four triazines were compared using glycine and cysteine as representative substrates containing amino and sulfhydryl groups. The unsubstituted compound, 2,4-dichloro-6-anilino-5-triazine, was found to be the least reactive in both cases, so results in the two series were compared to it as a standard. Reactivity with glycine

TABLE XII
RELATIVE REACTIVITIES OF THE *s*-TRIAZINE DERIVATIVES WITH CYSTEINE AND
GLYCINE AT 29° C. AND pH 7.0

| Derivative | Relative reactivity | |
|------------------|---------------------|----------|
| | Glycine | Cysteine |
| Unsub. | 1.0 | 1.0 |
| <i>p</i> -Chloro | 1.5 | 1.3 |
| <i>m</i> -Chloro | 1.7 | 1.5 |
| <i>o</i> -Chloro | 2.1 | 1.9 |

(Table XII) was found to decrease in the order *o*-chloro > *m*-chloro > *p*-chloro > unsub. with the *o*-chloro derivative being twice as reactive as the unsubstituted compound. The relative reactivity of the four compounds with cysteine was found to follow the same order found with glycine with minor quantitative differences. Thus the *o*-chloro derivative was found to react with cysteine 1.9 times faster than the unsubstituted compound and 2.1 times faster with glycine. The *meta* and *para* isomers were found to possess intermediate reactivity in both cases, with the *meta* isomer slightly faster.

DISCUSSION

MECHANISM OF ACTION

The first physiological response that was detected on treatment of conidia of *N. sitophila* with the triazines was rapid initial uptake followed by release of part of the accumulated material back into the medium. There is no satisfactory explanation for this except to assume that the permeabilities of the spore membranes were altered in such a way that free outward movement of materials, including part of the triazine, was permitted. These compounds can be extracted quantitatively from dilute aqueous solution by hydrocarbon solvents, so it seems unlikely that they would diffuse back into the aqueous phase unless associated with lipid or phospholipid globules released from the spores through the action of the toxicant. The pyridine reagent is capable of reacting with the triazines in organic media, so association with lipids would not prevent the development of color. An alternative theory would require selective concentration of the triazine in the aqueous phase of the spores followed by its release in true solution on disruption of the plasma membranes. In view of the solubility relationships of these compounds, this explanation appears to be untenable.

It is also possible that the release of triazine is an artifact caused by enhancement of the color developed in the analytical procedure by compounds released from the spores. Amino acids and peptides are known to do this. However, the reagent used in the analytical procedure was saturated with glycine in order to obtain maximum color development, so unless

some compound occurs in the spores which is much more effective than glycine in increasing color intensity, this possibility would appear to be eliminated. In any event the observed phenomenon must be ascribed to the outward movement of materials from the spores in response to the action of the triazine, since filtrates from untreated spores do not affect color intensity. This was given further confirmation by the finding that the *o*-chloro derivative can also cause the release of the radioactive phosphorus from spores grown on a medium containing $P^{32}O_4^{=}$. Thus all of the evidence indicates that disorganization of the cell membrane is one of the primary effects of the triazines at sub-lethal concentrations. This could probably occur through reaction of these compounds with sulfhydryl and amino groups of membrane proteins during diffusion into the cytoplasm. Extensive alkylation of side chain and terminal groups on protein molecules with a large heterocyclic radical would be expected to change their polarities, and hence their associations with each other, as well as with structural components such as the phospholipids. This structural disorganization could then result in a loss of selective permeability, and permit the free outward diffusion of protoplasmic constituents.

The shape of the curve obtained by plotting percentage uptake against time of exposure depends upon the amount of triazine present. At a dose of 500 μ g. per gram of spores, uptake is very rapid and a maximum is reached after which the triazine content of the external solution slowly increases. However in the presence of 1,000 μ g. of triazine per gram of spores, the curve reaches a maximum and then declines to a minimum. Following this, the net uptake again increases although at a much slower rate than occurs initially. This has been observed in a large number of cases, and although the minimum is not always distinct, a lag period is always observed in which the amount of triazine in solution remains constant or increases before net uptake by the spores is resumed.

These phenomena can be explained if it is assumed that the lipids in the spores which are associated with the triazine differ in degree of attachment to fixed nondiffusible structures within the spores, so that some of them can diffuse outward while others remain immobile after the selective permeability of the membrane is lost. Thus the rapid initial uptake would represent a period in which the triazine is associating with lipids and reacting chemically with spore constituents. Sometime during this period permeability would be altered and triazine in association with lipids would commence to move outward. When the rate of outward movement became equal to the rate of uptake, the net change would be zero, and the curve would reach a maximum. When the outward movement exceeded the uptake, the amount of triazine in the medium would increase. However, when the supply of diffusible material became exhausted, the rate of outward movement would diminish, and the rate of uptake by the immobile

structures would become equal to it at the minimum of the curve, and finally exceed it.

This phenomenon occurs only when the amount of triazine exceeds 500 $\mu\text{g.}$ per gram of spores. With smaller amounts of triazine the uptake reaches a maximum and then slowly decreases. There is no further reversal up to 100 minutes' exposure time. In these cases, the amount of triazine remaining in the external solution is relatively small, and the driving force responsible for continued uptake by the fixed structures would be correspondingly diminished. However, at a concentration of 1,000 $\mu\text{g.}$ per gram, about 20 per cent of the triazine remains in solution at the maximum compared to 5 per cent at 500 $\mu\text{g.}$ per gram. Thus continued uptake by the immobile spore structures would be favored by the comparatively high concentration of chemical in the external environment. The possibility that the distribution of triazine between spores and water is determined by concentration is indicated by the observation that spores containing 450 $\mu\text{g.}$ per gram slowly released 5 to 10 per cent of the material taken up when suspended in distilled water. In the presence of a large amount of triazine in the aqueous phase, net uptake would have been positive.

These observations show that uptake of the triazines by conidia of *N. sitophila* is a complex process involving changes in the permeability of the cell membranes, the outward diffusion of materials, and equilibrium effects. Furthermore, uptake appears to be associated with chemical combination of the triazine with intracellular metabolites since only 15 to 30 per cent of the material removed from solution can be recovered by extracting the spores after exposures of 1 to 20 minutes' duration.

These results appear to refute the theory (7) that the action of toxicants of this class can be explained by their ability to alter membrane permeability, since all the spores subjected to these treatments were able to germinate. The assumption that injury to the membrane causes the lethal effect seems very plausible, since any chemical entering the spore would have to traverse the membrane. If it were sufficiently reactive to destroy membrane integrity by combining with structural components containing amino and sulfhydryl groups, it might be expected to kill the cell through interference with selective transport systems. However, since doses which caused release of triazine and intracellular phosphorus did not have significant effects on either germination or respiration it must be concluded that continuous maintenance of selective permeability is not as critical to survival as had been supposed.

Even though exposure to potential doses of triazine of 1,000 $\mu\text{g.}$ per gram of spores fails to inhibit germination, it is possible to obtain inactivation by consecutive treatments with the toxicant and estimate ED₅₀ values on a spore weight basis. The average result of 1,530 $\mu\text{g.}$ per gram obtained for the *o*-chloro derivative is representative of the innate toxicity

of these compounds to conidia of *N. sitophila*. Thus the triazines are not comparable to penicillin in effectiveness (14), but are intermediate between dichloronaphthoquinone and mercury (12). Their high apparent toxicities must therefore be ascribed to the large amounts that can be taken up in relation to their concentration in aqueous solution. In the slide-germination test, the ED₅₀ of the *o*-chloro derivative was found to be 0.56 μ g. per ml. If the amount taken up is *ca.* 1,700 μ g. per cm.³ of conidia as indicated by innate toxicity and density (10) determinations, the ratio of the concentration in water to the concentration in spores is 1:3,000. Under these conditions only 5 per cent of the compound would be utilized.

Evidently the triazine must be present in large excess relative to the amount taken up to cause accumulation of lethal amounts by spores. This is indicated by the fact that a concentration of 0.2 μ g. per ml. does not impair germination although the compound is present in 7.5 times the amount required to inhibit germination of 50 per cent of the spores if it were all accumulated. The triazine is apparently partitioned between the spores and aqueous phase in such a way that uptake is favored by increasing the concentration in the medium. However, distribution between spores and water cannot be explained entirely on the basis of partition theory since the system is slow to reach equilibrium, and part of the material accumulated evidently reacts with constituents of the protoplasm.

RELATION BETWEEN STRUCTURE AND ACTIVITY

In addition to information on the mechanism of action of the triazines, these investigations have thrown some light on reasons why compounds with halogen atoms substituted in the benzene ring are more effective biologically than the unsubstituted 2,4-dichloro-6-anilino-*s*-triazine. These compounds differ in their order of reactivity with glycine and cysteine so that *o*-chloro > *m*-chloro > *p*-chloro > unsub. Apparently the substitution of a chlorine atom in the benzene ring decreases electron density around the carbon atoms in the triazine ring and renders them more susceptible to attack by nucleophilic compounds such as glutathione. This is an inductive ($-I_s$) or field effect, since the degree of activation diminishes progressively as the halogen is moved away from the triazine ring. If a mesomeric ($+M$) effect were involved, it would be expected that chlorine substitution in the benzene ring would deactivate the chlorine on the triazine ring toward nucleophilic substitution so that the order of reactivity would be *m*-chloro > unsub. > *o*-chloro > *p*-chloro. *Meta* substitution would be least effective in decreasing reactivity since a resonance isomer cannot be written in which the benzene and triazine rings are conjugated. It can be assumed, therefore, that halogen substitution produces ($-I_s$, $+M$) activation where $-I_s > M$.

The differences in chemical reactivity are not very great and, in the case of *N. sitophila* at least, they are not the major cause of differences in

biological activity. Thus the *o*-chloro derivative is 35 per cent more reactive with cysteine than the *p*-chloro derivative but the ED₅₀ value on a spore weight basis is only 10 per cent lower, and this difference is significant only at the 5 per cent level. Larger differences in performance might be expected between compounds where groups such as *p*-cyano, *p*-methoxyl, etc., are substituted in the benzene ring, since the activating effects would be different in sign and much larger differences in chemical reactivity would be expected. It seems likely that an optimum degree of reactivity must exist for compounds of this class, since a highly reactive derivative might hydrolyze in the environment before it could become effective, while a relatively unreactive material might not be able to compete with essential metabolites at rates greater than they can be replaced. In the paper by Schuldt and Wolf (16), a number of compounds are described which might provide examples of these types, but it is not possible to assess the importance of these effects without more information on their relative reactivities and the amounts which can be taken up by spores. In the group of compounds under current investigation, differences in reactivity are apparently unimportant although this may not be true for all species of fungi.

In experiments with conidia of *N. sitophila*, the amount of these materials which can be taken up by spores is a more critical factor than reactivity in determining apparent toxicity by the glass slide-germination procedure. In this technique the spores are immersed in solutions which in some cases contain 7.5 to 52.5 times the amount of triazine required to inhibit germination, so the amount which is taken up rather than the supply is limiting.

Substitution of a chlorine atom in the *ortho* position of the benzene ring of 2,4-dichloro-6-anilino-*s*-triazine increases cumulative uptake by 30 per cent when conidia of *N. sitophila* are exposed to successive doses of triazine, while *para* or *meta* substitution increases it by 80 per cent. However, when the weight of spores is very low in comparison to the amount of triazine, uptake tends to reach a limiting value and accumulation of the *para* isomer is favored. Thus the order of uptake at a low concentration of spores is *p*-chloro > *m*-chloro > *o*-chloro > unsub. which is the same order obtained for apparent toxicity.

The reasons for these differences in uptake have not been fully investigated. Rich and Horsfall (13) believe that the toxicological properties of any functional group can be improved by incorporating it into a molecule where the partition coefficient between water and lipids is large. This could possibly be a factor here, since the *para* isomer is less soluble in water than the *ortho* isomer so that its preferential uptake would be favored on the basis of partition theory. It is evident then, that uptake rather than chemical reactivity is the dominating factor in determining the order of toxicity of these compounds to conidia of *N. sitophila*. However, this might not necessarily apply to all species of fungi since Schuldt and Wolf (16)

have found the *ortho* isomer to be superior to the *para* isomer for the control of foliage diseases caused by the late blight and early blight organisms.

ACKNOWLEDGMENT

The authors would like to thank Dr. Lawrence P. Miller for data obtained on the release of radioactive phosphorus from conidia of *N. sitophila* on treatment with the *s*-triazine.

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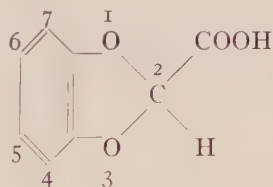
THE PREPARATION AND PLANT PHYSIOLOGICAL ACTIVITY OF 1,3-BENZODIOXOLE-2-CARBOXYLIC ACIDS

P. W. ZIMMERMAN, WILLIAM RICHARD SMITH, EDWARD A. PRILL,
AND A. E. HITCHCOCK

SUMMARY

1,3-Benzodioxole-2-carboxylic acid (BCA) and three chloro-substituted derivatives were prepared and tested for plant hormone-like activity. When applied to tomato plants as test objects, BCA and its 4,6-dichloro-substituted derivative caused epinasty of leaves, curvature of stems, proliferation of stem tissue, modification of leaves, parthenocarp, formation of adventitious roots, and stunting or killing with the higher concentrations. Though not so active from a concentration standpoint as 2,4-dichlorophenoxyacetic acid (2,4-D), the plant responses were similar. Threshold concentrations required in aqueous sprays for the different responses were as follows: modification of leaves, 0.001 per cent; epinasty, 0.005 per cent; bending and proliferation of stems, 0.05 per cent; and inhibition, 0.5 per cent. The 4,6-dichloro derivative had about the same plant physiological activity as BCA. The 5-chloro derivative was slightly less active. There were varying degrees of susceptibility among the 21 different species of plants treated.

The molecular configuration of 1,3-benzodioxole-2-carboxylic acid (BCA) is illustrated in the following formula:



The purpose of this paper is to report this new type of nucleus for plant growth regulators. Though not as active as 2,4-D on several species of plants, BCA, having a different nucleus, represents a new group of compounds which are interesting and may have practical value.

METHODS AND MATERIALS

The following species were used as test objects: basil (*Ocimum Basilicum* L.), bluegrass (*Poa annua* L.), carrot (*Daucus carota* L.), chickweed [*Stellaria media* (L.) Cyrillo], chickweed, mouse-ear (*Cerastium vulgatum* L.), clover, sweet (*Melilotus* sp.), *Coleus Blumei* Benth., dock (*Rumex* sp.), *Galinsoga* sp., *Geranium* sp., grape, Concord (*Vitis labruscana* Bailey), lamb's quarters (*Chenopodium album* L.), *Oxalis* sp., *Panicum* sp., peppergrass (*Lepidium* sp.), peppermint (*Mentha piperita* L.), pigweed (*Amaran-*

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thus retroflexus L.), pokeweed (*Phytolacca* sp.), *Potentilla* sp., sweet potato (*Ipomoea batatas* Lam.), and tomato (*Lycopersicon esculentum* Mill.).

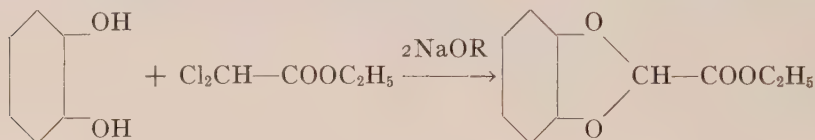
BCA and derivatives were dissolved in alcohol or neutralized with triethanolamine and then made up to volume with water. Solutions with known concentrations were applied to test plants as sprays (12), with brushes, or bacteriological loops (5, 12), and by injection into the stems (4, 13). Solutions were also applied to the soil in which the plants were growing (four-inch pots) so that the compound could be absorbed by the roots (12).

PREPARATION OF COMPOUNDS

The intermediary compounds were purchased or synthesized. The pyrocatechol and ethyl dichloroacetate were obtained from Eastman Kodak Co. The 4-chloropyrocatechol was prepared by chlorination of pyrocatechol with sulfuryl chloride in ethyl ether solution as described by Willstätter and Müller (9). The 3,5-dichloropyrocatechol was made from salicylaldehyde by chlorination as described by Biltz and Stepf (1) to give 3,5-dichloro-2-hydroxybenzaldehyde in good yield. The aldehyde group was then replaced by a hydroxyl group by means of alkaline hydrogen peroxide as described by Dakin (3).

BCA was previously prepared by Christiansen and Dolliver (2).

The main reaction in the formation of the benzodioxole compounds is illustrated by the following equation:



Pyrocatechol (or a substituted pyrocatechol) was reacted with 2 moles of a sodium alkoxide (NaOR) in anhydrous ethyl or isopropyl alcohol. Air was displaced by an inert gas, such as dry nitrogen, to prevent oxidation. The reactions with ethyl dichloroacetate took place rather slowly and yielded the ethyl esters of the desired acids. Actually, there were also unknown side reactions which consumed much of the starting materials causing low yields of esters. Furthermore, since the products of these side reactions tended to impede the isolation of the desired esters, a method of removing most of these and the unreacted pyrocatechol by precipitation with a barium compound was devised. The esters were purified by distillation *in vacuo* and were then saponified to yield the free acids. These are listed in Table I. The compounds were recrystallized from aqueous ethyl alcohol except for the 5-chloro compound which was recrystallized from cyclohexane. The details of the general method may be illustrated by the following specific preparation.

5-Chloro-1,3-benzodioxole-2-carboxylic acid. A three-liter round bottom flask set in a Glas-Col heating mantle was fitted with a reflux condenser, dropping funnel, and a tube for introducing dry nitrogen just above the surface of its contents. About 700 ml. of anhydrous isopropyl alcohol and 35 g. (1.52 moles) of metallic sodium were introduced and allowed to react until the sodium was dissolved. While the mixture was refluxing gently and was being continuously stirred, a slow continuous stream of dry nitrogen was introduced to prevent access of air. A solution of 110 g. (0.76 mole) of 4-chloropyrocatechol in approximately 400 ml. of anhydrous isopropyl alcohol was then added through the dropping funnel. Then a solution of

TABLE I
PROPERTIES OF 1,3-BENZODIOXOLE-2-CARBOXYLIC ACIDS AND ITS CHLORINATED ANALOGUES

| Compound | M.p., ° C. | Neutral calcd. | Equiva- lent found | Made from | B.p. of its ethyl ester ° C. at 3 mm. |
|--|---------------|-------------------|--------------------------|--------------------------|---|
| 1,3-Benzodioxole-2-carboxylic acid | 110 —111* | — | — | Pyrocatechol | 84–87 |
| 5-Chloro-1,3-benzodioxole-2-carboxylic acid | 137 —138.5 | 201 | 196 | 4-Chloropyrocatechol | 100–102 |
| 4,6-Dichloro-1,3-benzodioxole-2-carboxylic acid | 144.5–145 | 235 | 237 | 3,5-Dichloropyrocatechol | 106–109 (M.p., 61–62°) |
| Product of chlorination of BCA with hypochlorite | 150 —185 | — | — | — | — |

* Literature m.p. 107° to 108° (2).

120 g. (0.76 mole) of ethyl dichloroacetate in about 100 ml. of anhydrous isopropyl alcohol was added in the same manner. The mixture was refluxed gently in an atmosphere of dry nitrogen until it no longer showed an alkaline reaction (about three days). After cooling, the sodium chloride was filtered off and the liquid evaporated to about one-half its volume. To precipitate some of the by-products there were simultaneously added, with stirring, 100 ml. of triethylamine and a solution of 100 g. $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ in 300 ml. of warm water. The voluminous precipitate was removed by centrifugation and washed with ethyl alcohol. The combined supernatant liquid and the washings were reduced to a small volume by distillation under reduced pressure. The residue was diluted with water and extracted with ethyl ether. The ether extract was washed with dilute HCl, then with NaHCO_3 solution and dried over anhydrous Na_2SO_4 . After removal of the solvent and a little unreacted ethyl dichloroacetate, the ethyl ester of the



FIGURE 1. The effects of 1,3-benzodioxole-2-carboxylic acid on tomato plants. A. Terminal shoot from control plant (left) followed by one leaf and a terminal shoot 34 days after the entire plant had been sprayed with a 1 per cent solution, and (right) two leaves and a terminal shoot 27 days after the plant had been sprayed with a 0.05 per cent solution. B. Control plant (left) and plant treated 24 hrs. previously with 100 μ g. on one terminal leaflet. Note systemic effects, epinasty of leaves, and curvature of stem.

desired acid was distilled *in vacuo*. The free acid was readily obtained by boiling the ester with aqueous 1*N* NaOH until dissolved, then cooling and acidifying with dilute HCl. The compound was recrystallized from cyclohexane.

Product of chlorination of 1,3-benzodioxole-2-carboxylic acid with hypochlorite. A general method of chlorination with hypochlorite (6) was applied to a preformed benzodioxole compound. A mixture of 5 g. (0.033 mole) of 1,3-benzodioxole-2-carboxylic acid dissolved in 35 ml. of 1*N* NaOH and 100 ml. of water and 198 ml. of a 5 per cent sodium hypochlorite solution (0.132 mole) was held at 20° C. for one hour. On acidifying with HCl the product was precipitated. Since it melted over a wide range (150° to 185° C.), it was obviously a mixture of chlorinated acids. It probably contained some of the 5,6-dichloro acid.

RESULTS AND DISCUSSION

BCA was tested for plant physiological activity. The solutions were applied in several different ways, and an effort was made to determine threshold concentrations for given responses and the most effective concentrations for other effects. The principal responses observed were epi-

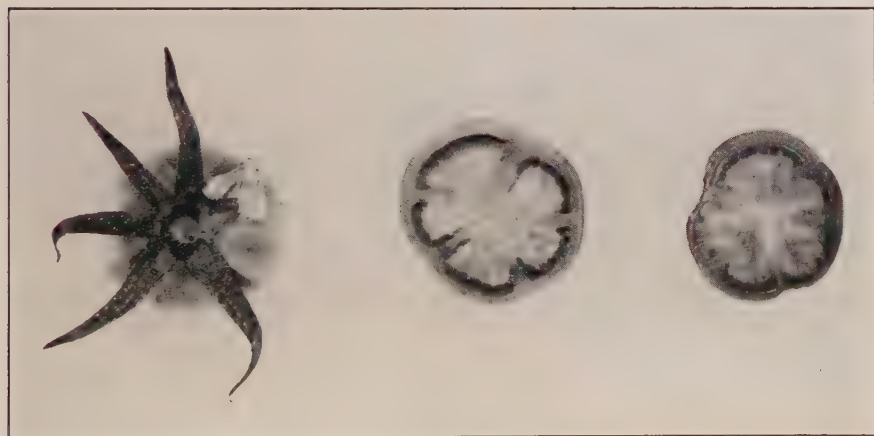


FIGURE 2. Parthenocarpy of tomato 34 days after a small plant had been sprayed with a 0.01 per cent solution of 1,3-benzodioxole-2-carboxylic acid. Left, the upper end showing flower petal under the calyx. Middle, cut surface showing seedlessness. Right, cross-section through middle of fruit.

nasty of leaves, modification of leaves (Fig. 1 A), curvatures of stems (Fig. 1 B), inhibition or killing of leaves and plants, parthenocarpy (Fig. 2), and induction of roots (10). The effective concentrations of BCA and the degree of response by treated tomato plants are recorded in Table II. A concentration of 0.001 per cent of BCA caused little or no epinasty of tomato

TABLE II
DEGREE* OF RESPONSE INDUCED ON TOMATO PLANTS WITHIN A TEN-DAY PERIOD FROM
DIFFERENT AMOUNTS OF 1,3-BENZODIOXOLE-2-CARBOXYLIC ACID

| Methods of application, solution in per cent | Modification of leaves | Leaf and/or stem curvatures | Stunting or killing | Rooting of tomato leaf cuttings from dip method | Parthen- ocarp resulting from spray |
|--|---------------------------|--------------------------------------|---------------------------|--|---|
| Spray, per cent | | | | | |
| 0.0001 | o | o | o | o | o |
| 0.001 | X | o-X | o | o | o |
| 0.006 | XX | XX | o | o | ? |
| 0.01 | XX | XX | o | o | X |
| 0.05 | XXX | XXX | X | o | X |
| 0.125 | XXX | XXX | X | X | X |
| 0.5 | XXXX | XXXX | XX | XXXX | XX |
| 1.0 | XXXX | XXXX | XXXX | XXX | — |
| 2.0 | XXXXX | XXXXX | Killed | Killed | — |
| 0.01 Ml. bacteriological loop to tomato leaflet | | | | | |
| 0.125 | X | X | — | — | — |
| 0.5 | X | XX | — | — | — |
| 1.0 | XX | XXX | — | — | — |
| 2.0 | XXX | XXXX | — | — | — |
| 10 Ml. applied to soil | | | | | |
| 0.001 | X | XX | — | — | — |
| 0.006 | XX | XX | — | — | — |
| 0.015 | XXX | XX | — | — | — |
| 0.03 | X | XX | XX | — | — |
| 0.06 | — | XXX | XX | — | — |
| 0.125 | — | XXXX | XXX | — | — |
| 0.50 | — | XXXXX | Killed | — | — |

* o=no response; X=slight response; XX=moderate response; XXX=considerable response; XXXX=pronounced response; XXXXX=very pronounced response.

leaves, but the new growth which appeared after the plants were sprayed became modified (Fig. 1 A). Also 10 ml. of this concentration applied to the soil caused modification of tomato leaves. A concentration of a 0.125 per cent solution applied to a single tomato leaflet by means of a 0.01-ml. bacteriological loop caused the new leaves to become modified. These concentrations are approximately 10 times greater than required for 2,4-D. Similar differences hold also for the other responses which can be induced by the BCA and 2,4-D.

Twenty-one species of plants were sprayed with a 1 per cent solution of BCA by means of an atomizer to determine comparative resistance or susceptibility. The results are listed in Table III. As measured by bending of stems and leaves, the species were affected in the following order: tomato, *Galinsoga*, lamb's quarters, and chickweed. As measured by the amount of killing, the species were affected in the following order: *Oxalis*, pokeweed, tomato, basil, and clover. In comparison, 0.1 per cent of 2,4-D

TABLE III

RESULTS FROM SPRAYING 21 SPECIES OF PLANTS WITH A 1 PER CENT CONCENTRATION OF 1,3-BENZODIOXOLE-2-CARBOXYLIC ACID

| Plant species | Bending of leaves and stems within 24 hrs. | Necrosis or injury to leaves and stems | Remarks |
|-----------------------------|--|--|--|
| Basil | o-X* | XX | Tip of shoot and flower buds killed. |
| Bluegrass | o | Tips of leaves X | Continued growth. |
| Carrot, wild | X | o | Recovered quickly. |
| Chickweed | X | o | New growth after treatment showed modified leaves. |
| Chickweed (mouse-ear) | XX | — | |
| Clover | Curling | XX | Recovered and made new growth in 10 days. |
| Coleus | X | X | New leaves after treatment modified. Terminal bud became enclosed. |
| Dock, sour | Curled leaf X | X | Recovered. |
| Galinsoga | XXX | o | |
| Geranium | Curling | X | Regrowth within 10 days. |
| Grape (Concord) | Curled X | X | Young leaves killed. |
| Lamb's quarters | XXX | X | Recovered quickly. |
| Oxalis | o | Killed | |
| Panicum grass | o | o | Resistant species. |
| Peppergrass | X | o | Recovered quickly. |
| Peppermint | X | Curling X | Recovered quickly. |
| Pigweed | o | o | No damage. |
| Pokeweed | o | XXX | Field-tested plants were badly damaged with 1% solution. |
| Potentilla | o | o | Resistant. |
| Sweet potato (var. Triumph) | o | o | Plant was inhibited for a time. New leaves were modified. |
| Tomato | XXXX | XX | Recovered and made new growth in 10 days. |

* See Table II footnote for degree of response.

would kill practically all of the species which are listed in Table III, except grasses (10). However, BCA might be a more effective herbicide if formulated as emulsions or dissolved in oil.

BCA effectively modified the leaves of tomato plants when applied as a foliage spray, when added to the soil in which the plants were growing, or where one leaflet was treated with a given concentration from a bacteriological loop. The tomato plants were not badly inhibited after being sprayed with a 0.5 per cent solution of BCA and later produced flowers and fruit. Plants which were treated on September 28 with this concentration still showed modification of leaves on November 16 when they were 3 ft. in height. Also the flowers of these plants showed enlarged ovaries before the petals opened. As the ovaries enlarged, the petal of the flower remained between the sepal and the ovary, in contrast with the normal way of cling-

ing to the blossom end of the fruit (Fig. 2). When the chemical solution was applied directly to the flower cluster as the petals opened, 0.01 per cent was effective for parthenocarp. Also parthenocarp was induced when 10 ml. of a 0.01 per cent solution was applied to the soil of a four-inch pot in which the tomato plant was growing. A 0.1 to 0.5 per cent solution applied with a brush to flower clusters was effective for inducing parthenocarp of tomato.

Adventitious roots were induced along the stems of tomato plants after they had been sprayed with BCA at 0.1 per cent or higher concentrations. Tomato leaf cuttings dipped into solutions containing from 0.5 to 1.0 per cent BCA produced more roots than the controls treated with water only.

TABLE IV

APPROXIMATE THRESHOLD CONCENTRATION (PER CENT) OF 4,6-DICHLORO-1,3-BENZODIOXOLE-2-CARBOXYLIC ACID REQUIRED TO INDUCE DIFFERENT RESPONSES ON TOMATO PLANTS WHEN APPLIED BY VARIOUS METHODS

| Responses | Threshold concn. (%) when applied by | | |
|------------------------|--------------------------------------|---------------------------------|------------------------------------|
| | Foliage sprays | Bacteriological loop (0.01 ml.) | 10 Ml. of solution applied to soil |
| Modification of leaves | 0.0025 | 0.06 | 0.005 |
| Epinasty of leaves | 0.0025 | 0.03 | 0.005 |
| Stem bending | 0.005 | 0.03 | 0.005 |
| Inhibition to killing | 0.50-2.00 | — | 0.06-0.125 |

The control cuttings averaged 13 roots per cutting, as compared with 21 roots for cuttings dipped into a solution of 0.5 per cent and 18 roots for the 1.0 per cent solution. Two per cent solutions damaged the cuttings. Geranium stem cuttings rooted profusely after being dipped into a 0.123 per cent solution of BCA as compared with only a few roots on control cuttings.

Tomato plants were stunted or killed when sprayed with solutions containing 0.5 per cent to 2 per cent of BCA. Similarly, a solution containing 0.5 per cent of BCA stunted or killed tomato plants when 10 ml. were applied to the soil of a four-inch pot. No attempt has been made to formulate BCA for herbicidal purposes, but the results (Table III) indicate that effective preparations could be made.

Since 4,6-dichloro-1,3-benzodioxole-2-carboxylic acid was not soluble at a concentration greater than 0.005 per cent while the triethanolamine salt was soluble at 0.5 per cent, most of the treatments were made with the salt. The results listed in Table IV show approximate threshold concentrations required to induce the various responses. The substituted and nonsubstituted molecules have approximately the same degree of physiological activity. In this respect the BCA acid differs from phenoxyacetic acids, in which case substitution of chlorine greatly increases activity.

However, some other substitutions might increase the activity of BCA.

5-Chloro-1,3-benzodioxole-2-carboxylic acid was slightly less effective than the dichloro-substituted molecule, but it also caused epinasty, stem bending, and modification of leaves when plants were sprayed with a 0.05 per cent solution.

The product of chlorination of BCA with hypochlorite was found to have very low hormone-like activity but was more phytotoxic than the other three compounds. It was nearly insoluble and difficult to apply to plants unless formulated with toxic carriers such as alcohol or acetone. The triethanolamine salt of this compound also had very low activity.

The structure of BCA is somewhat related to that of phenoxyacetic acid except that the connection between the benzene ring and the acetic acid residue is through two oxygen atoms instead of one. As a consequence of the extra oxygen containing ring, the spatial positions of the carboxyl group and the α -hydrogen atom are necessarily fixed above and below the plane containing the rings, whereas, in the case of phenoxyacetic acid, the carboxyl group and the α -hydrogen atom can each assume a variety of spatial positions both in and outside the plane of the benzene ring. This may be of interest in connection with theories on the structural requirements for activity, such as the theory of the three-point attachment of the compound to the substrate through the benzene ring, the carboxyl group, and an α -hydrogen atom (8). In the present compounds the rigid fixed configuration may perhaps make difficult the proper postulated fit to the substrate molecule.

It may be pointed out that the benzodioxole nucleus, which is also important in compounds that are synergists for pyrethrin insecticides, exists under a considerable strain (7), and this may possibly have some connection with activity in the present compounds.

The plant physiological activity of compounds having a benzene ring can often be changed by substitution of halogen into various positions in the ring. This has been used to change the activity of phenoxy and benzoic acids (11). The chlorinated BCA acids which were tested did not show greater plant hormone activity than the unsubstituted acid, although they were often more phytotoxic.

To the knowledge of the authors, 1,3-benzodioxole-2-carboxylic acid and its derivatives have not been previously reported as plant growth regulators. Due to the small amount of the compound available, they have not been thoroughly tested for growth-regulating effects or their possible use as plant herbicides. However, enough observations have been made to indicate that their effects are similar to those of 2,4-D except for the fact that higher concentrations of BCA are required to bring about comparable results.

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Note

IMPROVED METHOD OF HARVESTING HOUSE FLY HEADS FOR USE IN CHOLINESTERASE STUDIES

HERBERT H. MOOREFIELD

The current interest in the organophosphates as insecticides has resulted in the publication of numerous researches involving cholinesterase assays. Many of these investigations, entailing the use of insect-derived enzyme preparations, have been *in vitro* studies directed at gaining basic information on mode of action or establishing molecular structure-toxicity correlations. As the head of the house fly (*Musca domestica* L.) has been shown (1) to be very rich in this enzyme, and flies are readily adaptable to mass laboratory rearing, this has become a popular source of insect cholinesterase. In all of this work, however, it appears to be common practice to decapitate the flies individually by hand. This is a time-consuming procedure as well as a monotonous task.

In this laboratory, it has been found expedient to freeze the adult flies at -10° C. and fragment the body divisions and appendages by vigorous agitation in a closed container or merely lightly grind them together with the hand over a rough surface. The separated heads are then easily isolated by sifting through a screen (100 openings per square inch) which will exclude the thoraces and abdomens. The wings are then removed with a gentle air stream and the legs readily pass through a second screen of narrower mesh. Alternately, the entire procedure can be carried out equally well in a single operation within a refrigerated room by using appropriate nested brass sieves. The inclusion of irregular, hardened objects, such as rubber stoppers, in the uppermost container with the intact flies will facilitate the decapitation. It has also been noted that the specific activity of the fly head cholinesterase is unaffected by the freezing procedure, and the heads can be satisfactorily stored below 0° C. for at least one week without loss of enzymatic activity.

With this method, several thousands of heads can be collected within a few minutes. This technique should be especially helpful in fractionation and purification studies, wherein large quantities of concentrated fly cholinesterase are required.

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1888

QUANTITATIVE DETERMINATION OF AMINO ACIDS BY PAPER CHROMATOGRAPHY

CLARK A. PORTER, DAVID MARGOLIS, AND PAMELA SHARP

SUMMARY

A method is described for the quantitative determination of amino acids and amides separated on two-dimensional paper chromatograms. The amino compounds are reacted on the paper with a ninhydrin solution containing 8-hydroxyquinoline in an ethanol-saturated atmosphere at 60° C. for 30 minutes. These conditions were found superior to others tested when evaluated by yield and uniformity of color. The colored products are eluted from the paper by 50 per cent ethanol containing 0.025*M* phosphate buffer at pH 7.0 and their absorbances determined photometrically. The color is stable in this solution for at least 24 hours following elution.

Data are presented to illustrate the reproducibility of the method when applied to standard amino acid solutions of known concentration and to the free amino acids and amides of plant extracts.

INTRODUCTION

During investigations necessitating the quantitative determination of amino acids in plant extracts, the paper chromatographic methods tried were not found entirely satisfactory. One-dimensional methods (1, 3, 4) were not useful because all of the components of the extracts could not be resolved. A two-dimensional method involving elution of the developed ninhydrin color was essential but the color developmental method employed (8) was not satisfactory because of erratic results. The reason for this was not apparent but during the investigation of possible variables a method was developed that has given reproducible results in this laboratory. The method will be described in detail since it is more rapid and less tedious in certain respects than other published methods (2, 7, 9).

THE QUANTITATIVE METHOD

CHROMATOGRAPHY

Two-dimensional descending chromatography is carried out on 18½" × 22¼" papers using aqueous phenol in the first direction and a *n*-butanol:acetic acid:water mixture in the second direction. Table I indicates the amount of solvent used for two papers and the approximate running time for Whatman No. 1 and No. 3 paper at 22° to 24° C.

Whatman No. 1 or Whatman No. 3 chromatography grade filter papers are satisfactory but the latter gives better resolution of amino acids in

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TABLE I
VOLUME OF SOLVENT REQUIRED FOR TWO PAPERS (18½"×22¼") AND THE
APPROXIMATE DURATION OF CHROMATOGRAPHY FOR WHATMAN
NO. 1 AND NO. 3 PAPERS AT 22° TO 24° C.

| Paper | Solvent | | | |
|---------------|---------|------|-------------------------------------|------|
| | Phenol | | <i>n</i> -Butanol-acetic acid-water | |
| | Ml. | Hrs. | Ml. | Hrs. |
| Whatman No. 1 | 90 | 30 | 125 | 30 |
| Whatman No. 3 | 175 | 24 | 200 | 24 |

plant extracts. Either paper is used without preliminary washing or buffering.

The solvents employed for chromatography are Mallinckrodt liquefied phenol (88 per cent without preservative; labeled "For Chromatography") in a 4:1 (v/v) mixture with water, adjusted to an apparent pH of 5.5 to 5.8 (glass electrode) with sodium hydroxide and *n*-butanol:acetic acid:water (90:10:29, v/v/v). Deionized water is used in the preparation of both solvents. The phenol solvent is used within two days of preparation and the *n*-butanol:acetic acid:water solvent is made up fresh before use.

Large paraffin-lined wooden boxes with vapor-tight lids are used as containers for chromatography and are maintained at constant temperature in control cabinets. Each chromatography box has five Pyrex troughs and accommodates ten papers. Two papers are held in each trough by binding their edges between two glass rods held tightly together at the ends by rubber bands. By holding the papers in this manner a more even run is obtained since the origins on all papers are equidistant from the solvent surface. The papers are supported by glass rods so that they will not hang directly on the edge of the solvent troughs. Shallow enameled pans containing the appropriate solvent are placed in the bottom of the box to saturate the enclosed space during a run. The papers are placed in the troughs and the solvent added immediately. Elimination of an equilibration period prior to solvent addition has not been found detrimental to separations or color yields. At the end of a run the papers are removed from the troughs after securing them to the supporting rods with spring-type wooden clothes pins and cutting them free from the binding rods. The supporting rods and attached papers are then placed in the hood to dry.

The papers are dried overnight following exposure to each solvent. This is accomplished at room temperature in a hood having a strong draft. During and following drying of the solvents from the papers it is absolutely essential that the papers not be exposed to vapors from formic acid, hydrochloric acid, collidine, lutidine, ammonia and similar substances, which will produce erratic results in color development.

ASSAY FOR AMINO ACIDS AND AMIDES

Color development is carried out using 1 per cent ninhydrin in absolute ethanol containing 0.1 per cent 8-hydroxyquinoline ('Baker Analyzed' Reagent). The solution is applied to the papers from a pipette and they are heated for 30 minutes at 60° C. in an atmosphere saturated with ethanol vapor. A cabinet similar to that described by Thompson *et al.* (8) was used initially for heating the papers but later was replaced by a stainless steel tank submerged in a constant-temperature water bath. The papers are removed and dried in a current of air.

The individual colored spots are identified and outlined in pencil, cut into strips, weighed on a Roller-Smith balance, further cut into pieces approximately 1 cm.² in area, and placed in test tubes. To each tube is added 5 ml. of 50 per cent ethanol containing 0.025*M* phosphate buffer (K_2HPO_4 - NaH_2PO_4) at pH 7.0, the tubes are covered with Parafilm, and shaken by hand. The tubes are shaken again approximately one-half hour before determining the absorbance of the eluted color. This time interval allows any suspended paper fragments to settle to the bottom of the tubes. The readings are made with a Beckman spectrophotometer using a 10-mm. light path at any time from 6 to 30 hours following the introduction of the eluting solution. The absorbance of all the amino acids except proline and asparagine are read at 570 $m\mu$. Proline is read at 430 $m\mu$ and asparagine is read at 360 $m\mu$.

Background corrections are made by cutting a comparable section of paper from an amino acid-free area, eluting it and measuring the absorbance of the solution at the appropriate wave lengths. One such area is taken near the phenol front and used for proline, leucine, phenylalanine, valine, and γ -aminobutyric acid. A second area is taken near asparagine and used for this amide, for glutamine, and for the remainder of the amino acids. A background-correction factor based upon absorbance per mg. of paper is used to adjust the value for each amino acid spot to determine its corrected absorbance. If the background appears to vary measurably in different localities one's own judgment and experience as to the necessary number of areas utilized for correction must be used. The use of other solvent systems may necessitate using areas other than those indicated because of different distribution of the amino acids on the paper.

Standard amino acid solutions, at a number of known concentrations, are subjected to the above procedure. A standard curve may be prepared for each amino acid by plotting the absorbance values against the corresponding concentrations of the amino acid. As an alternative and recommended procedure, a regression equation is calculated (6, pp. 108-111) for each amino acid using the appropriate absorbances. Substitution of the absorbances obtained for an unknown in the regression equation gives the mg. of amino acid in the 5-ml. volume in which the color is eluted.

In order to assure the desired accuracy, triplicate chromatograms are prepared for each determination. Periodically, standard amino acid solutions are analyzed as a check on the constancy of controlled conditions of development. These determinations have given values within the expected range of variation.

DISCUSSION AND EVALUATION OF THE METHOD

CHROMATOGRAPHY

The chromatographic procedures described in this paper have been found satisfactory for the resolution of the amino acids in a variety of plant extracts. The use of liquefied phenol without preservative as a replacement for the crystalline preparation was found to be more convenient and less hazardous. The solvent prepared with this phenol has consistently shown an apparent pH of 4.5 before adjustment with sodium hydroxide. Phenol containing preservative is less satisfactory because of its high and variable acidity. Slight temperature changes during chromatography are of no significance as both solvent combinations are single phase systems. Precise temperature control is not essential if temperature fluctuation is not more than would be expected under ordinary laboratory conditions as the wooden boxes are well enough insulated to resist all but unusual temperature changes. Whatman No. 3 paper has been of particular advantage for obtaining satisfactory resolution of the amino compounds when dealing with extracts containing large amounts of interfering substances. Recently a grade of this paper prepared specially for chromatography has become available. It is superior to the regular grade because of uniformity of texture and composition that result in more equivalent running time between individual sheets of the same and different packages. It also has a very low heavy metal content, which is important when utilizing it for quantitative amino acid analyses. Both grades of Whatman No. 3 paper have been used successfully but the special grade has consistently yielded less variable results.

COLOR DEVELOPMENT

Preliminary work to determine the most suitable conditions for color development employed one-dimensional chromatograms of individual amino acids. These chromatograms were prepared by development in the same direction, first with aqueous phenol and then with *n*-butanol-acetic acid-water; the papers being dried well before exposure to the second solvent. This gave chromatograms comparable to two-dimensional ones in that they had been exposed to both solvents and the amino acids had migrated on the paper. Therefore, the effect of any residual solvent would not be ignored in the selection of the most satisfactory conditions of color development. All of the later critical work was carried out by the two-dimensional descending method.

TABLE II

COMPARISON OF THE ABSORBANCES RESULTING FROM THE REACTION OF SELECTED AMINO ACIDS WITH NINHYDRIN IN DIFFERENT ATMOSPHERES AT 60° C. FOR 30 MINUTES

| Amino acid | Atmosphere* | | | | | | Relative humidity | |
|---------------|---------------|----------------------------|-------------------------------------|------|-----------------|--|-------------------|------|
| | "Ethanol-air" | "Ethanol-CO ₂ " | "Ethanol-CO ₂ quiescent" | Air | CO ₂ | | 35% | 100% |
| | | | | | | | | |
| Aspartic acid | .110 | .093 | .101 | .158 | .078 | | .110 | .041 |
| Serine | .688 | .513 | .608 | .449 | .500 | | .536 | .469 |
| Alanine | .786 | .764 | .814 | .676 | .658 | | .622 | .483 |
| Asparagine | .670 | .620 | .620 | .495 | .525 | | .370 | .275 |
| Proline | .642 | .667 | .654 | .856 | .897 | | .779 | .883 |

* Complete description of experimental atmospheres given in text.

The atmosphere inside the color development tank was altered in preliminary work to find the conditions that would give the highest and most reproducible color yields. It had been found that color development at 60° C. for 30 minutes was most suitable for all atmospheres and therefore these conditions remained constant throughout. The atmospheres compared were air and CO₂ alone and each in conjunction with ethanol saturation, and relative humidities of 100 and 35 per cent. The latter condition was included to determine the effect of an intermediate moisture content, and for comparison with results obtained at this relative humidity at 20° C. (9). Saturation of the cabinet with ethanol was obtained by placing absolute ethanol in the bottom and resulted in the "ethanol-air" atmosphere. The "ethanol-CO₂" atmosphere was obtained by bubbling CO₂ through the ethanol. The "ethanol-CO₂ quiescent" condition was arrived at by flushing out the tank with CO₂ to replace the air and securing the cabinet without further addition of CO₂. A relative humidity of 100 per cent was realized by replacing the ethanol in the cabinet with distilled water. The cabinet was conditioned to 35 per cent relative humidity by bubbling air through a water-glycerine mixture (11) before it entered the tank.

The data in Table II are the comparative absorbances of selected amino

TABLE III

REPRODUCIBILITY OF COLOR DEVELOPMENT IN ATMOSPHERES OF "ETHANOL-AIR" AND "ETHANOL-CO₂"

| | "Ethanol-air" | | "Ethanol-CO ₂ " | |
|--------------------------|---------------|---------|----------------------------|---------|
| | Serine | Alanine | Serine | Alanine |
| Absorbance* | .630 | .715 | .515 | .718 |
| Coefficient of variation | 6.2 | 5.5 | 17.2 | 10.8 |

* Each value is the average of 30 determinations obtained from 5 replicate chromatograms developed on 6 different days.

acids developed in the test atmospheres. No one method gave superior results for all amino acids but "ethanol-air" gave the most satisfactory results for the bulk of amino acids when evaluated on the bases of color yield and uniformity. A more critical evaluation comparing "ethanol-air" with "ethanol-CO₂" was made using serine and alanine as the test amino acids. It is apparent from the data in Table III that the papers developed in "ethanol-air" gave higher reproducibility of color than those developed in "ethanol-CO₂."

It was observed that the color intensity and quality of ninhydrin-reacted amino acids were changed by the varying environmental conditions, but all amino acids were not affected alike. This finding is in agreement with Wellington (10), and it is similarly concluded that a single amino acid used as an internal standard is not a valid indicator of changes that may occur in all amino acids.

TABLE IV

EFFECT OF 8-HYDROXYQUINOLINE UPON YIELD AND REPRODUCIBILITY OF THE ALANINE-NINHYDRIN COLOR DEVELOPED IN AN ATMOSPHERE OF "ETHANOL-AIR"

| | Ninhydrin | | Ninhydrin + 8-hydroxyquinoline | |
|----|------------|--------------------------|--------------------------------|--------------------------|
| | Absorbance | Coefficient of variation | Absorbance | Coefficient of variation |
| 1* | .745 | 0.81 | .911 | 1.1 |
| 2 | .706 | 4.2 | .884 | 1.0 |
| 3 | .776 | 1.03 | .879 | 2.3 |
| 4 | .742 | 4.7 | .891 | 1.9 |

* Absorbance values in rows 1, 2 and 3 are averages of 6 replicate chromatograms of alanine on individual papers; row 4 is a composite of the 18 replicates showing the variability between papers.

An attempt was made to control further the conditions of color development by incorporating a buffer into the ninhydrin solution. This was not successful because of the very slight solubility of common buffer salts in absolute ethanol. A buffer made from 2-amino-2-hydroxymethyl-1,3-propandiol + HCl ("Tris"), which is soluble in ethanol, was also investigated for utility but produced a high background and low color yields. Since it has been shown that certain cations interfere with the development of ninhydrin color (5), it was thought that some variability might result from differences in the cation content of different lots of paper. In an effort to remove possible interfering cations by chelation, 8-hydroxyquinoline was incorporated into the ninhydrin solution. Table IV illustrates the results obtained using alanine as a test amino acid. The use of 8-hydroxyquinoline yielded considerably higher color intensity and less variability. Comparison was made between "ethanol-air" with 8-hydroxyquinoline in the ninhydrin and the original method employing "ethanol-

CO₂." Table V shows the data obtained for several amino acids. It is apparent that color intensity is increased and that the reproducibility within a single set of determinations, as well as between determinations on different days, is much higher when 8-hydroxyquinoline is employed.

Comparable color development is obtained by employing 0.1 per cent 8-hydroxyquinoline in the phenol during chromatography. Its incorporation into phenol is advantageous as it results in an apparent pH of 5.8 which obviates adjustment with sodium hydroxide. The background cor-

TABLE V

REPRODUCIBILITY OF COLOR DEVELOPMENT FOR AMINO ACIDS IN ATMOSPHERES OF "ETHANOL-CO₂" AND "ETHANOL-AIR" WHEN THE LATTER IS EMPLOYED IN CONJUNCTION WITH 8-HYDROXYQUINOLINE IN THE NINHYDRIN

| Amino acid | | "Ethanol-CO ₂ " | | | "Ethanol-air" | | |
|---------------|----|----------------------------|------------|--------------------------|-------------------|------------|--------------------------|
| | | No. of replicates | Absorbance | Coefficient of variation | No. of replicates | Absorbance | Coefficient of variation |
| Alanine | 1* | 4 | .316 | 10.4 | 4 | .410 | 4.6 |
| | 2 | 3 | .339 | 5.6 | 3 | .394 | 2.3 |
| | 3 | — | — | — | 3 | .403 | 1.2 |
| | 4 | 7 | .327 | 4.9 | 10 | .402 | 2.0 |
| Aspartic acid | 1 | 4 | .062 | 30.6 | 4 | .340 | 3.8 |
| | 2 | 3 | .094 | 19.2 | 3 | .352 | 2.3 |
| | 3 | — | — | — | 3 | .366 | 3.8 |
| | 4 | 7 | .078 | 29.5 | 10 | .353 | 3.7 |
| Glutamic acid | 1 | 4 | .236 | 11.4 | 4 | .445 | 1.3 |
| | 2 | 3 | .301 | 6.6 | 3 | .445 | 0.9 |
| | 3 | — | — | — | 3 | .484 | 3.7 |
| | 4 | 7 | .268 | 17.2 | 10 | .458 | 4.8 |
| Glycine | 1 | 4 | .226 | 17.7 | 4 | .426 | 4.0 |
| | 2 | 3 | .288 | 4.2 | 3 | .396 | 1.8 |
| | 3 | — | — | — | 3 | .417 | 2.6 |
| | 4 | 7 | .257 | 17.2 | 10 | .413 | 3.6 |

* Absorbances for rows 1, 2 and 3 are averages of replicate two-dimensional chromatograms determined on 3 different days; row 4 is a composite of the data in rows 1, 2 and 3.

rections for proline and asparagine are less when 8-hydroxyquinoline is incorporated into the phenol, rather than the ninhydrin, because it and its complexes are washed from the paper by the second solvent. However, 8-hydroxyquinoline cannot be used satisfactorily in the phenol when Whatman No. 3 paper is employed because the dicarboxylic amino acids migrate too far in the phenol direction; aspartic acid and glutamic acid overrun serine and threonine respectively. This condition can be overcome if, prior to chromatography, the paper is washed with dilute calcium hydroxide and then thoroughly washed with deionized water.

STABILIZATION OF THE ELUTED COLOR

Using the described solvent combination, the ninhydrin colors eluted in 50 per cent ethanol faded with time and had to be read within four hours to obtain reliable results. The color could be stabilized to some degree by storing in the refrigerator which then allowed the colors to be developed and eluted one day and read the next. Since the observed insta-

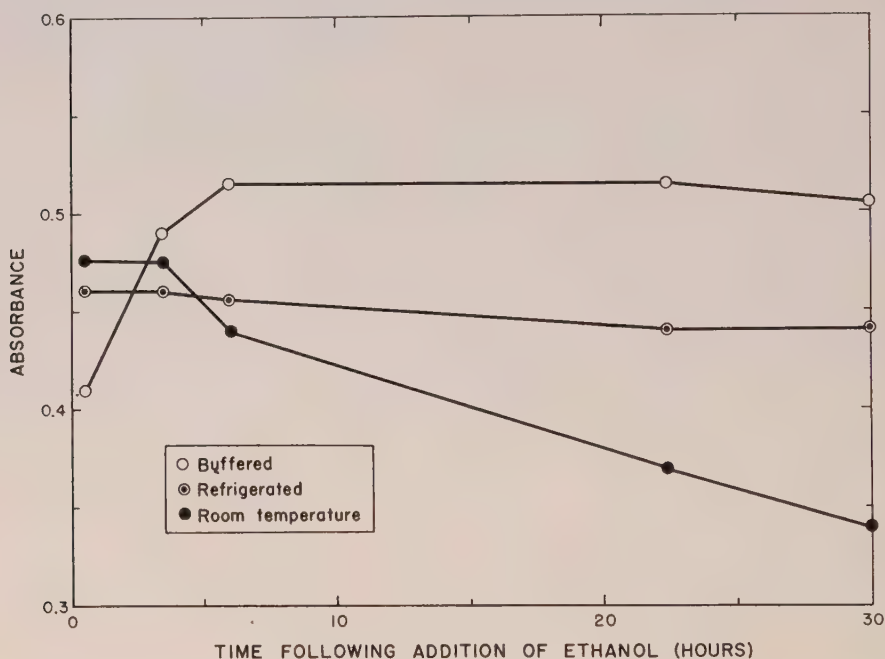


FIGURE 1. Comparison of ninhydrin color stability in 50 per cent ethanol at room temperature and under refrigeration, and in 50 per cent ethanol containing 0.025M phosphate buffer (pH 7.0) at room temperature.

bility could be due to acidity caused by the second solvent, a phosphate buffer at pH 7.0 was incorporated into the eluting solution to eliminate this condition. Figure 1 compares ninhydrin color stability in 50 per cent ethanol at room temperature and in the cold, and in 50 per cent ethanol containing 0.025M phosphate buffer (pH 7.0) at room temperature. The color is eluted completely in 6 hours by the buffered ethanol and is stable for 24 hours more at room temperature. Its stability beyond this period has not been determined. The color in buffer is not adversely affected by daylight but the tubes probably should not be placed in direct sunlight. Routinely they are placed in a cabinet which exposes them to diffuse light only.

ABSORPTION CHARACTERISTICS OF PROLINE AND ASPARAGINE

Figure 2 shows the absorption spectra of proline and its background in the presence of 8-hydroxyquinoline in 50 per cent ethanol containing 0.025*M* phosphate buffer (pH 7.0) at room temperature. The secondary maximum of proline at 430 $m\mu$ was selected for measurement because at this wavelength the background correction is small. This consideration is

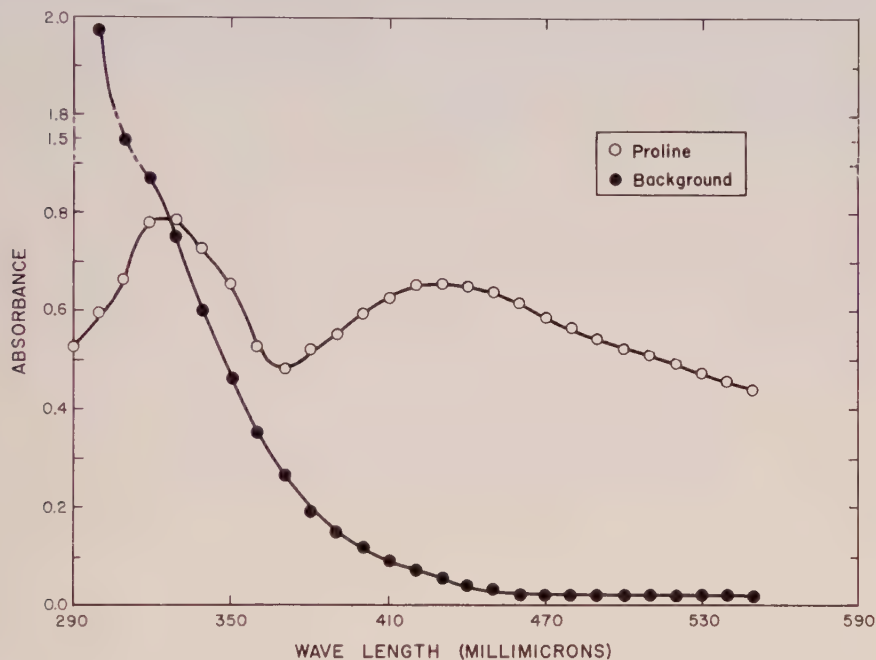


FIGURE 2. Absorption spectra of proline (corrected for background) and background, in the presence of 8-hydroxyquinoline, in 50 per cent ethanol containing 0.025*M* phosphate buffer (pH 7.0) at room temperature.

especially important when small amounts of proline are determined. If the major maximum at 330 $m\mu$ were used, a small error in the background correction could completely obliterate a small absorbance for proline. The same procedure is used for asparagine which has nearly equivalent maxima at 310 $m\mu$ and 360 $m\mu$. The 360 $m\mu$ maximum is used since the background correction is much less at this wavelength.

A number of attempts to elute, stabilize, and determine photometrically the blue color produced by the reaction of isatin with proline were unsuccessful.

EVALUATION OF THE METHOD

Table VI shows the reproducibility obtained using standard amino acid solutions of varying concentration. These values are averages of

TABLE VI
REPRODUCIBILITY OF REPLICATES OF STANDARD AMINO ACIDS CHROMATOGRAPHED
IN TWO DIMENSIONS AND DETERMINED BY THE DESCRIBED METHOD

| Amino acid | No. of triplicate determinations | $\mu\text{G.}$ | Absorbance | Coefficient of variation |
|-----------------------------|----------------------------------|----------------|------------|--------------------------|
| Alanine | 4 | 2 | .063 | 20.6 |
| | | 5 | .194 | 4.6 |
| | | 10 | .413 | 2.2 |
| | | 20 | .850 | 2.9 |
| γ -Aminobutyric acid | 5 | 2 | .038 | 23.7 |
| | | 5 | .116 | 4.3 |
| | | 10 | .242 | 3.7 |
| | | 20 | .494 | 5.9 |
| Asparagine | 3 | 2 | .073 | 28.8 |
| | | 5 | .105 | 17.1 |
| | | 10 | .158 | 10.8 |
| | | 20 | .265 | 4.5 |
| | | 40 | .478 | 1.0 |
| | | 80 | .905 | 2.8 |
| Aspartic acid | 4 | 2 | .033 | 57.6 |
| | | 5 | .085 | 21.2 |
| | | 10 | .172 | 8.7 |
| | | 20 | .348 | 3.2 |
| | | 40 | .698 | 1.4 |
| Glutamic acid | 4 | 2 | .051 | 37.3 |
| | | 5 | .122 | 16.4 |
| | | 10 | .241 | 8.7 |
| | | 20 | .478 | 5.0 |
| | | 40 | .954 | 3.4 |
| Glutamine | 3 | 5 | .057 | 24.6 |
| | | 10 | .129 | 8.5 |
| | | 20 | .272 | 2.2 |
| | | 40 | .559 | 1.1 |
| Glycine | 3 | 2 | .050 | 24.0 |
| | | 5 | .190 | 3.7 |
| | | 10 | .424 | 4.0 |
| | | 20 | .890 | 5.4 |
| Phenylalanine | 2 | 2 | .022 | 40.9 |
| | | 5 | .044 | 13.6 |
| | | 10 | .089 | 1.1 |
| | | 20 | .179 | 8.9 |
| Proline | 2 | 5 | .033 | 24.2 |
| | | 10 | .083 | 9.6 |
| | | 20 | .183 | 4.4 |
| | | 40 | .383 | 2.1 |
| Serine | 4 | 2 | .063 | 25.4 |
| | | 5 | .167 | 7.8 |
| | | 10 | .341 | 2.6 |
| | | 20 | .688 | 1.9 |
| Threonine | 4 | 2 | .046 | 28.3 |
| | | 5 | .130 | 8.5 |
| | | 10 | .268 | 2.9 |
| | | 20 | .546 | 2.0 |
| Valine | 5 | 2 | .037 | 29.7 |
| | | 5 | .129 | 6.2 |
| | | 10 | .283 | 3.2 |
| | | 20 | .591 | 3.6 |

TABLE VII

REPRODUCIBILITY OF TRIPPLICATE DETERMINATIONS OF THE FREE AMINO ACIDS OF TURKISH TOBACCO LEAVES BY THE STANDARDIZED METHOD

| Amino acid | μ G. | Absorbance | Coefficient of variation |
|-----------------------------|----------|------------|--------------------------|
| Alanine | 11.2 | .461 | 1.1 |
| γ -Aminobutyric acid | 49.6 | 1.254 | 9.6 |
| Arginine | 18.6 | .187 | 2.1 |
| Aspartic acid | 12.0 | .211 | 10.4 |
| Glutamic acid | 12.3 | .290 | 3.1 |
| Glutamine | 163.0 | 2.355 | 2.0 |
| Glycine | 5.7 | .220 | 9.5 |
| Leucine | 9.8 | .159 | 3.8 |
| Lysine | 9.2 | .109 | 5.5 |
| Serine | 40.1 | 1.371 | 5.6 |
| Threonine | 7.3 | .195 | 3.6 |
| Valine | 4.0 | .100 | 9.0 |

triplicate determinations carried out on the number of different days indicated. It is apparent from the data that the same degree of accuracy is not obtainable for all levels of a given amino acid; also the concentration for maximum accuracy is not the same for all. In general, measurements involving less than 10 μ g. are subject to relatively large error, while those in the range of 10 to 40 μ g. result in excellent reproducibility. Quantities greater than 40 μ g. can be determined with equivalent accuracy providing satisfactory chromatographic separations are obtained. Phenylalanine is worthy of special mention since it has an optimum range of determination around 10 μ g. and the variability increases rapidly when this range is exceeded. γ -Aminobutyric acid exhibits similar characteristics but its variability increases less rapidly.

Tables VII and VIII present examples of the reproducibility of determinations on extracts prepared from the 80 per cent ethanol soluble components of Turkish tobacco and barley leaves. These extracts were not

TABLE VIII

REPRODUCIBILITY OF 9 REPLICATE DETERMINATIONS OF THE FREE AMINO ACIDS OF BARLEY LEAVES BY THE STANDARDIZED METHOD

| Amino acid | μ G. | Absorbance | Coefficient of variation |
|-----------------------------|----------|------------|--------------------------|
| Alanine | 8.3 | .412 | 3.6 |
| γ -Aminobutyric acid | 9.1 | .215 | 6.0 |
| Asparagine | 70.8 | .826 | 3.9 |
| Aspartic acid | 3.9 | .047 | 21.3 |
| Glutamic acid | 11.1 | .239 | 12.1 |
| Glutamine | 30.8 | .415 | 4.3 |
| Lysine | 18.6 | .206 | 10.2 |
| Serine | 7.4 | .263 | 9.9 |
| Threonine | 6.4 | .184 | 7.1 |
| Valine | 11.5 | .318 | 5.3 |

subjected to ion-exchange or other techniques for the removal of inorganic and organic contaminants prior to chromatography. The variability in the determinations is in close agreement with the predicted variability based upon the data for equivalent amounts of amino acids determined in standard solutions. Notable exceptions are the values obtained for serine and glycine in the Turkish tobacco leaf extract. The reason for this discrepancy is the inability to obtain clear-cut separations of these two compounds when one or both occur in the concentration shown for serine. However, the reproducibility shown by these compounds in this case is acceptable.

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A COMPARATIVE STUDY OF THE AMINO ACID COMPOSITION OF COMMERCIAL SAMPLES OF A HIGH-PROTEIN AND A LOW-PROTEIN WHEAT FLOUR

RICHARD J. BLOCK AND RICHARD H. MANDL

SUMMARY

The amino acids yielded by hydrolysis of commercial samples of high-protein wheat flour (N=2.9 per cent) and low-protein wheat flour (N=2.0 per cent) were compared by means of quantitative paper chromatography.

The analytical results indicate that there are apparently significant differences in the amounts of cystine, valine, glutamic acid and proline in the total proteins of the wheat flours.

No significant differences were found in the remaining amino acids present in the two types of flour including *lysine*, the amino acid which is limiting in the proteins of wheat flour. Thus, when wheat flour supplies a significant portion of the total protein intake, foods made from high-protein flours would be expected to be superior to those prepared from low-protein flour.

It is generally accepted, since the original publications of Osborne and Mendel (from Mendel, 11) that lysine is the amino acid which limits the nutritive value of wheat proteins for growing animals. There is also considerable evidence that as the protein content of the cereal grains is increased by various means, the quantity of lysine and certain other essential amino acids per 100 g. of protein (16.0 g. of N) is decreased. Thus McElroy *et al.* (9) reported that their studies on Marquis wheat suggested a trend towards a decrease in the percentage of lysine in the protein with increasing levels of total protein in the wheat. Price (17) found that English wheat containing 1.58 per cent of N yielded 2.4 g. of lysine per 16.0 g. of N, while Manitoba wheat with 2.43 per cent of N yielded only 2.0 g. of lysine per 16.0 g. of N. Pence *et al.* (16) in a comparative study of the amino acid composition of glens prepared from 17 varieties of flour also suggested a similar decrease in the proportion of lysine in glens prepared from high-protein flour, although their results are not clear cut and contain a number of exceptions to the general statement. Miller *et al.* (12) reported no difference in the percentage of lysine in various wheat samples containing from 14.7 to 16.6 per cent of protein (N \times 6.25). More marked changes were found by Mitchell *et al.* (14) between high- and low-protein corn. They reported that U. S. Hybrid 13 with 7.32 per cent protein yielded 2.9 g. of lysine per 16.0 g. of N, while Illinois high-protein corn containing 20.04 per cent protein yielded only 1.8 g. of lysine per 16.0 g. of N. Similar results have been reported by Sauberlich *et al.* (18), Flynn *et al.* (5), Frey (6)

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and Miller *et al.* (13). The decrease in lysine was explained by the fact that high-protein corn contained a greater proportion of zein. Melhus *et al.* (10) found that varieties of teosinte containing from 20 to 23 per cent protein yielded only one-half as much lysine per 16.0 g. of N as did samples of corn containing 8.2 to 9.6 per cent protein. The low nutritive quality of teosinte has been confirmed by Yang *et al.* (19). Frey (7) reported that a variety of oats with 9.3 per cent protein contained 5.9 g. of lysine per 16.0 g. of N, while a 15.8 per cent protein oats yielded only 3.6 g. of lysine per 16.0 g. of N.

The evidence summarized above strongly suggests that there is less lysine in the proteins of high-protein cereal grains than in those of lower total protein content. This decrease in lysine of the proteins of the whole grain may be due to three possibilities: (A) a shift in the ratio of the amounts of germ proteins to endosperm proteins; (B) a relative increase in the quantity of the prolamines in proportion to the total proteins of the endosperm; and (C) an actual change in the amino acid pattern of the proteins. Suggestion (C) may be dismissed because of the prevailing opinion that identical proteins from varieties of the same organism do not change significantly in amino acid composition. Suggestion (B) is the one favored by Frey (6), Mitchell *et al.* (14), and Sauberlich *et al.* (18), to explain the changes in the lysine content of corn. If the same explanation holds for wheat, then one would assume that flour prepared from high-protein wheat should contain a lower percentage of lysine in its proteins than flour made from low-protein wheat. However, if this were not the case, then the results are better explained by suggestion (A).

Because from one-fourth to one-third of the total protein consumed in the United States is derived from white flour and not from whole wheat (2, p. 168; 3, p. 493), a comparative study of the amino acid composition of two commercial samples of white flour, differing in total protein content, was undertaken. The results indicate that there does not appear to be any significant difference in the lysine content of the proteins of standard commercial low-protein and high-protein white flour.

EXPERIMENTAL

Low-protein flour. The sample of low-protein flour was a vitamin enriched, 72 per cent, patent wheat flour sold under the brand name "Pillsbury's Best." It contained 2.0 per cent of N.

High-protein flour. A sample of high-protein flour was obtained from the Electric Steel Elevator, Duluth, Minnesota under the trade name "Energetic." The container also had on it the following descriptive phases: "bromated," "high gluten," "unenriched" and "bleached." Because of the notation "high gluten," an inquiry was made of the manufacturer whether wheat gluten had been added to the flour, and assurance was given that

no gluten had been added. The high-protein flour was prepared from 1956 wheat grown either in Minnesota or Montana. The degree of extraction of this flour was not made available to us but it is probably a 72 per cent extraction flour. It contained 2.9 per cent of N. These brands were chosen because of their availability and are assumed to be representative of high-protein and low-protein wheat flours.

Preparation of samples for hydrolysis. Because the large quantity of carbohydrates present in white flour usually causes considerable destruction of amino acids during acid and alkaline hydrolysis, part of the carbohydrates were removed in the following fashion (2, p. 14): 10 g. of each flour were triturated with 90 ml. of hot water until no lumps remained. Then the pH of the suspension was adjusted to 4.5 with 5*N* acetic acid and it was heated in a boiling water bath for 2 hours. At the end of the heating period, 180 ml. of cold water were added and the suspension was neutralized with 5*N* NaOH and cooled to 37° C. Then 25 ml. of fresh human saliva, clarified by centrifugation, were added. The starch was digested at 37° C. for 24 hours in the presence of a few ml. of toluene. At the completion of the digestion, the suspension was adjusted to pH 4.5 with 5*N* acetic acid and the insoluble protein was removed by centrifugation. The precipitate was thoroughly washed with hot water, acetone, hot benzene: absolute ethanol (95:5 v/v), ethanol and ether. The residue was then dried to constant weight in air.

Hydrolysis of the proteins. Five hundred mg. samples were hydrolyzed under reflux for 20 hours with 25 ml. of 6*N* HCl for all amino acids except tryptophan (2, p. 71). The latter was determined in hydrolyzates prepared by refluxing the samples with 14 per cent w/v Ba(OH)₂·8H₂O (2, p. 72).

After removal of the excess HCl and the insoluble humin, the quantity of nitrogen in the hydrolyzates was determined. These values were used to calculate the distribution of the amino acids in the proteins prepared from the high- and low-protein flours. This method of calculation would be expected to yield a higher¹ quantity of amino acids than that based on the hydrolysis of flours not digested with ptyalin because of the assumed decrease in the destruction of many amino acids during hydrolysis (2, p. 21) and the exclusion of any N lost in the humin. The quantity of N lost in the humin was, unfortunately, not determined. Under these conditions of hydrolysis it is probably less than 10 per cent of the total N.

Amino acid analysis. The quantities of amino acids in the hydrolyzates were estimated by the maximum density of the spots on one-dimensional paper chromatograms (1; 2, p. 71; 3, p. 440). The reproducibility of this procedure is approximately ±10 per cent (2, p. 85) and this compares

¹ Analysis of an hydrolyzate of 1,000 mg. of the high-protein flour prepared by refluxing the flour with 100 ml. of 6*N* HCl gave 2.3 g. of lysine per 16.0 g. of N rather than the value of 2.7 shown in Table I.

favorably with the usual chemical and microbiological methods (4). However, the maximum density method is not as reproducible as the ion exchange column method (2).

RESULTS

The analytical results obtained are presented in Table I. It will be seen that except for cystine, valine, glutamic acid and proline, the pattern of amino acids in the proteins of the two flours analyzed is similar. The analytical values reported by Kraut *et al.* (8) and by Nunnikhoven (15) on

TABLE I
DISTRIBUTION OF AMINO ACIDS IN HIGH-PROTEIN AND IN LOW-PROTEIN WHEAT FLOURS

| Amino acid | G. of amino acid per 16.0 g. of N in the filtered hydrolyzates | | | | |
|---------------|--|--------------------------------|------------------------------------|------------------------------------|---|
| | High-protein flour (2.9% N), g. | Low-protein flour (2.0% N), g. | 9.8% Protein flour [Kraut (8)], g. | 8.9% Protein flour [Kraut (8)], g. | 75% Extraction Manitoba II flour [Nunnikhoven (15)], g. |
| Arginine | 4.3 | 4.4 | 3.6 | 3.6 | 3.5 |
| Histidine | 2.5 | 2.7 | 1.3 | 1.3 | 2.1 |
| Lysine | 2.7 | 2.7 | 2.5 | 2.5 | 2.1 |
| Tyrosine | 2.5 | 2.9 | 4.0 | 3.5 | 3.2 |
| Tryptophan | 0.8 | 0.8 | 1.4 | 1.2 | — |
| Phenylalanine | 6.3 | 6.4 | 4.9 | 4.9 | 5.3 |
| Cystine | 2.8 | 1.9 | 4.8 | 4.0 | 2.0 |
| Methionine | 1.5 | 1.5 | 1.0 | 0.7 | 1.5 |
| Serine | 4.1 | 4.4 | 4.7 | 4.7 | 4.3 |
| Threonine | 3.7 | 4.0 | 2.1 | 2.5 | 2.8 |
| Leucine | 7.4 | 8.6 | — | — | 6.7 |
| Isoleucine | 4.7 | 4.7 | — | — | 3.5 |
| Valine | 4.2 | 5.5 | 3.7 | 2.9 | 3.5 |
| Glutamic acid | 37.5 | 31.2 | 32.2 | 29.3 | 32.8 |
| Aspartic acid | 3.9 | 4.0 | 4.0 | 3.8 | 4.2 |
| Glycine | 3.0 | 3.2 | 3.4 | 3.4 | 3.4 |
| Alanine | 3.2 | 3.6 | — | — | 2.7 |
| Proline | 12.5 | 9.1 | 11.1 | 10.3 | 11.4 |
| Amide N | 3.8 | 3.6 | 4.3 | 4.4 | 3.9 |

acid hydrolyzates of starch-containing white flour are also given. Kraut *et al.* (8) employed partition chromatography on starch columns for the majority of the amino acids (*cf.* 2, p. 117) while the results of Nunnikhoven (15) on a single sample of flour are included to show the pattern of amino acids obtained by the method of ion exchange column chromatography (*cf.* 2, p. 119). The values for cystine reported by Kraut *et al.* (8) are probably high as these figures were calculated from the difference between the total sulfur and the methionine S of his preparations. Many of the other discrepancies seen in Table I may be ascribed to actual variations in the amino acid composition of the proteins of the samples of flour, to hydrolytic losses and to errors in the methods of analysis (*cf.* 2, p. 85; 4).

The smaller quantity of cystine found in the high-protein flour may have been the result of processing (addition of bromate and bleaching) or to actual differences in the composition of the proteins (*cf.* 12). The larger quantities of glutamic acid and of proline found in the flour of higher protein content by Kraut *et al.* (8) and in this study indicate that, as the gluten content of the endosperm is increased, the prolamin fraction may increase at a more rapid rate. This increase in prolamin is similar to that reported to occur in corn (5, 6, 13, 14, 18). However, the quantity of lysine in the proteins of both flours was the same and because lysine is the limiting essential amino acid in white flour, it appears that foods made from high-protein wheat flours are nutritionally as satisfactory on an isonitrogenous basis as those prepared from low-protein flours and are nutritionally superior when equal amounts of flour are employed.

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EFFECT OF FUNGICIDES ON OXYGEN CONSUMPTION AND VIABILITY OF MYCELIAL PELLETS¹

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SUMMARY

Mycelial pellets of *Alternaria oleracea*, *Aspergillus niger*, *Monilinia fructicola* and *Myrothecium verrucaria* obtained from shaken cultures were treated with various fungicides and the effect on oxygen consumption and viability observed. The uptake of fungicides and their effect on viability were also determined.

The quantity of fungicide required, on an external concentration basis, to reduce oxygen consumption by one-half was less, in general, than the quantity required to prevent growth in 50 per cent of the pellets. Cadmium, zinc, cerium, sodium arsenate and cycloheximide had little or no effect on oxygen consumption or viability. The toxicity of silver and mercury, as measured by reduction in viability, doubled for each 3-fold increase in time of exposure.

The uptake of toxicants by the pellets was slow but continued for many hours so that with the more toxic materials ED₅₀ values could be obtained. Metal ions such as zinc and cerium were taken up in very small amounts and consequently had no effect on viability. Comminution of pellets of *A. niger* caused silver to be taken up three times as rapidly even though it reduced oxygen consumption greatly. Comminution had no effect on the uptake of silver by pellets of *A. oleracea*.

The oxygen consumption of the pellets with endogenous substrates was approximately 3 μ l. per mg. of fresh weight during 5 hours. This rate was increased only 50 per cent or less when sucrose was added to the medium.

In comparison with the results previously obtained (9, 10, 11, 13) with the conidia of the same species it is apparent that the endogenous oxygen consumption of the pellets, on a fresh weight basis, is only one-half to one-third that of the conidia, while in the presence of sucrose it is only one-third to one-sixteenth that of the spores. With conidia, unlike the pellets, reduction in viability was a more sensitive response to toxicants than was oxygen consumption. Uptake of toxicants by the pellets is much slower than by the conidia. However, ED₅₀ values for silver and dichlone are more or less comparable for pellets and conidia.

INTRODUCTION

The control of mycelial growth despite its importance is not nearly as far advanced as that of spore germination. Also there is considerable information regarding the reaction of fungus spores to fungitoxicants. Recently a comparative study was made of the effect of fungicides on the oxygen consumption and germination of fungus spores (9). It therefore was of interest to make a similar survey of the response of mycelium to

¹ These investigations were conducted in cooperation with the United States Atomic Energy Commission, Contract AT(30-1)-788. A preliminary report was presented before the American Phytopathological Society, Cincinnati, Ohio, December 1956 (7).

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fungicides. Since information has been obtained on the uptake of fungicides by spores (10, 11, 13) some studies have also been made on the uptake by mycelium. Finally the data presently obtained on the responses of the mycelium have been compared with that previously obtained for the spores.

Since the investigations of Kluyver and Perquin in 1933 (6) demonstrated the merits of "Mycelkügelchen" or mycelial pellets, from shaken submerged cultures, they have become of increasing interest and were selected for the present study. Mycelial pellets have been found to give more uniform responses than mycelial mats or fragments; they are more readily handled and are beginning to be used in studies on fungicides (1, 3, 5, 6, 16).

MATERIALS AND METHODS

Mycelial pellets were obtained from shaken submerged cultures of fungi used previously (9), namely: *Aspergillus niger* van Tiegh, *Alternaria oleracea* Milbraith, and *Monilinia fructicola* (Wint.) Honey. *Myrothecium verrucaria* (Alb. & Schw.) Ditm. ex Fr. was used in some limited preliminary tests. Attempts to obtain suitable pellets of *Neurospora sitophila* (Mont.) Shear & Dodge, on various media were unsuccessful. The pellets were produced by seeding 50 ml. of nutrient media in 200-ml. Erlenmeyer flasks with the appropriate spores and placing in a rotary action shaker operating at about 200 oscillations per minute at room temperature. The spores were obtained from culture media prepared as described previously (9, 12). Several different media were employed, the most common being a synthetic one prepared according to Burkholder and Sinnott (1) and containing mineral salts, glucose, asparagine, trace elements and vitamins. Other media used were glucose-yeast extract (1) and potato dextrose (14). *Monilinia fructicola* was also cultured on a dextrose-malt medium (1.5 per cent dextrose, 3.5 per cent malt extract) and on 10 per cent orange juice from canned frozen preparations.

The pellets were used when from 3 to 7 days old. They were separated from the culture medium by filtration, washed about three times with distilled water, spread out on filter paper to dry off for about 15 minutes and then approximately equal lots placed in weighing bottles. The quantity of pellets used per flask in the respirometer studies varied from about 30 to 300 milligrams depending on the fungus and experimental lot. All data have been expressed on a fresh weight basis. Dry weight determinations were also made on additional duplicate samples.

An 18-unit rotary Barcroft-Warburg microrespirometer was operated at 30° C. for measuring oxygen consumption. The techniques used were similar to those reported previously (9). The pellets were suspended in a solution which, after the addition of fungicide, had a volume of 2 ml., and

a sucrose concentration of 1 per cent. Each variable was set up in duplicate and the runs terminated 5 hours after introducing the fungicide. The pellets were then recovered, washed and centrifuged twice, and samples of 10 pellets from each flask plated out on potato dextrose agar in Petri dishes and examined for growth after several days. The agar was acidified with lactic acid to inhibit the growth of bacteria (14, p. 30).

The fungicides comprised the water-soluble materials previously studied (9), namely silver nitrate, copper sulfate, mercuric chloride, cadmium chloride, zinc acetate, cerous sulfate, sodium arsenate, cycloheximide (3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutaramide), and malachite green. A volume of 0.2 ml. of fungicide solution was added to the side arm of the microrespirometer. A range of concentrations was employed, usually varying in steps of $\sqrt{10}$. The fungicide was introduced into the main flask containing the pellets, 1 hour after the beginning of the test.

The effective dose of the toxicant necessary to reduce oxygen consumption or number of pellets growing by 50 per cent, i.e., the ED₅₀ value, was estimated from dosage-response curves plotted on logarithmic-probability paper. ED₅₀ values are expressed as micrograms of applied metal ion or organic compounds per milligram fresh weight of pellets in conformity with the previous paper (9).

Tests were also conducted in which pellets varying in weight from 3 to 150 mg. were suspended in 10 ml. of various concentrations of toxicant in 15-ml. centrifuge tubes with conical bottoms for periods of 10 minutes, 1 hour, and 6 hours. At the end of each period 10 pellets were removed, centrifuged and washed twice and plated out for a determination of viability. Dose was expressed as above.

Studies on the uptake of fungicides were made using ions of Ag¹¹⁰, Zn⁶⁵, Ce¹⁴⁴, and C¹⁴-labeled dichlone (2,3-dichloro-1,4-naphthoquinone) and employing the techniques described earlier (10, 11, 13). The amount of fungicide taken up is expressed as micrograms per gram of fresh weight of pellet in conformity with prior uptake studies (10, 11). The distinction between dose expressed as micrograms uptake per gram of pellets and external solution applied per milligram of pellets is to be noted.

RESULTS

NUTRIENTS EMPLOYED FOR GROWING PELLETS

Numerous comparisons were made of the growth of pellets of *Aspergillus niger* and *Alternaria oleracea* in the synthetic, potato dextrose, and yeast extract media. In general there was little difference in yield. With both species, the pellets tended to grow larger in the yeast extract media. The synthetic medium was generally considered most satisfactory. On this medium both species produced more or less compact and uniform pellets

varying but little in size within a given lot. *A. niger* pellets were whitish with a pale yellow cast and ranged from about 0.5 to 1.5 mm. in diameter in different lots. *Alternaria oleracea* pellets were at first greyish-white and after several days turned olive-brown or even black while the size varied from about 1 to 3 mm. in diameter. The mean fresh weight per pellet was approximately 1 mg. for *A. niger* and 2 mg. for *A. oleracea*. Limited comparisons indicated that on an equivalent weight basis the pellets produced on the three different media did not differ significantly in oxygen consumption.

Monilinia fruticola produced practically no pellets in the synthetic medium. The pellet production on potato dextrose, glucose-yeast extract, and orange juice was erratic with a tendency toward a few large pellets ranging widely in size from 1 to 10 mm. diameter. Yields on the dextrose-malt medium were somewhat better and the pellets somewhat smaller and more uniform in size. The pellets were light greyish-brown in color and much less compact than those of the other species.

TABLE I
DRY WEIGHT OF MYCELIAL PELLETS EXPRESSED AS PERCENTAGE OF FRESH WEIGHT

| Fungus | Mean % dry wt. | Coefficient of variation | |
|-------------------------------|----------------|--------------------------|-------|
| | | Replicates | Tests |
| <i>Alternaria oleracea</i> | 11.1 | 9.9 | 40.0 |
| <i>Aspergillus niger</i> | 10.7 | 7.4 | 32.4 |
| <i>Monilinia fruticola</i> | 8.0 | 10.0 | 49.1 |
| <i>Myrothecium verrucaria</i> | 10.4 | 4.0 | 54.5 |

DRY WEIGHT OF PELLETS

Dry weights of pellets expressed as per cent of fresh weight were obtained on duplicate samples in approximately 20 tests on each of three fungi and in 3 tests for *Myrothecium verrucaria*. An analysis of these data is presented in Table I. It will be seen that the mean dry weight is between 10 and 11 per cent for three of the fungi and 8 per cent for *Monilinia fruticola*. Within a given test the replicates agree closely with a coefficient of variation of 10 per cent or less. In fact if the most variable pair of replicates is eliminated with each of the first three fungi, i.e. 5 per cent of the data, the coefficients of variation are reduced respectively to 6.7, 5.7, and 6.3 per cent. It is thus possible to obtain an adequate expression of the dry weight of a given lot of pellets on a very limited sample. However, the variation is considerably more on different lots or tests. This is presumably due primarily to the different extent of drying out before obtaining the fresh weight and to a lesser extent on inherent variations in different lots of pellets.

TABLE II
MEAN OXYGEN CONSUMPTION BY PELLETS OF FOUR SPECIES OF FUNGI WITH
AND WITHOUT SUCROSE

| Fungus | Microliters O ₂ per mg. pellets in 5 hours | | |
|-------------------------------|---|-----------------|------------|
| | Without sucrose | With 1% sucrose | L.S.D., 5% |
| <i>Alternaria oleracea</i> | 2.87 | 4.10 | 0.85 |
| <i>Aspergillus niger</i> | 2.99 | 4.57 | 1.37 |
| <i>Monilinia fruticola</i> | 2.92 | 3.21 | 0.42 |
| <i>Myrothecium verrucaria</i> | 3.79 | 5.60 | 1.14 |

ENDOGENOUS AND EXOGENOUS OXYGEN CONSUMPTION

Several tests were made on the effect of sucrose upon oxygen consumption and the data are summarized in Table II. It is apparent that the presence of sucrose resulted only in a 40 to 50 per cent increase for *Alternaria oleracea*, *Aspergillus niger* and *Myrothecium verrucaria* and was without significant effect on *Monilinia fruticola*. Glucose also did not bring about a significant increase in oxygen consumption by pellets of *M. fruticola*.

In the previous article (9, Table I) there is given a similar comparison for spores. It should be pointed out that "exogenous" as used in the previous paper refers to the total oxygen consumption in the presence of sucrose, hence a direct comparison may be made between the two sets of data. (More commonly "exogenous" refers to the increased oxygen consumption resulting from added substrate.) In comparison with the pellets, the spores of three of the fungi, namely *Alternaria oleracea*, *Monilinia fruticola* and *Myrothecium verrucaria*, in the presence of 1 per cent sucrose increased their oxygen consumption, respectively, 2.3, 2.5, and 10.5-fold. A direct comparison of these data on spores with all those available for the pellets in the presence of 1 per cent sucrose is given in Table III. Here it will be seen that the spores are much more active than are pellets on a fresh weight basis with the oxygen consumption in the presence of sucrose ranging from about 3 to 16 times as great for the spores.

The possibility was considered that the relatively lower activity of the

TABLE III
MEAN OXYGEN CONSUMPTION ON A FRESH WEIGHT BASIS IN 1% SUCROSE FOR
PELLETS AND SPORES OF FOUR SPECIES OF FUNGI

| Fungus | Microliters O ₂ per mg. tissue in 5 hours | |
|-------------------------------|--|--------|
| | Mycelial pellets | Spores |
| <i>Alternaria oleracea</i> | 4.00 | 17.5 |
| <i>Aspergillus niger</i> | 4.10 | 13.4 |
| <i>Monilinia fruticola</i> | 3.16 | 50.7 |
| <i>Myrothecium verrucaria</i> | 3.20 | 31.0 |

pellets might be due to their compactness or other morphological differences which would hinder penetration. As was noted earlier the mycelium of *Neurospora sitophila* does not produce compact discrete pellets similar to the other fungi. However, on modified Fries medium (12) small loosely packed and partially discrete pellets were produced. On modified Horowitz medium (12) even more open and more or less interwoven masses of mycelium were produced, while in the synthetic medium of Burkholder and Sinnott (1) a single large wad of cottony-like mycelium more or less similar to a mycelial mat from a stationary culture was produced. These three lots of mycelium were compared for oxygen consumption endogenously and in the presence of 1 per cent sucrose. Amounts of mycelium ranging from 100 to 150 mg. fresh weight were used. It was necessary to tear apart the mycelial mat of the third lot to obtain samples. At the end of a 5-hour test it was found that the first lot of mycelium most closely resembling pellets had consumed 3.46 μ l. of oxygen per mg., the second lot 3.10 μ l. per mg. and the third lot of more or less typical mycelium only 1.93 μ l. per mg., based on the mean of endogenous consumption and that in the presence of sucrose. The increased oxygen consumption in the presence of sucrose ranged from 38 per cent for the first lot of more or less discrete pellets to no difference for the mycelial mat. It will also be noted that the value of 3.46 μ l. oxygen for the first lot of mycelium is comparable to that for pellets of other species as given in Table II. The dry weights calculated as per cent of fresh weight ranged from 8.0 for the first lot of mycelium to 11.7 for the third lot, so on a dry weight basis the differences in oxygen consumption would be even more marked. These results would indicate that as the mycelial forms become more pellet-like they increase in physiological activity rather than lose activity because of compactness. This is in accord with the findings of others (1, 6).

EFFECT OF TOXICANTS ON OXYGEN CONSUMPTION AND VIABILITY

The results of the effectiveness of the various toxicants in reducing oxygen consumption and inhibiting subsequent growth of the mycelial pellets of the three species are summarized in Table IV in terms of ED₅₀ values. For many of the chemicals, e.g. cadmium, zinc, and cerium, the highest concentrations used were without effect on either oxygen consumption or viability. For the other toxicants, with one marked exception, the ED₅₀ values for oxygen consumption were equal to or lower than those for viability. The exception is the effect of cycloheximide on the mycelial pellets of *Monilinia fructicola* where the viability response is markedly more sensitive than oxygen consumption. The marked resistance of *Aspergillus niger* pellets to copper both as regards oxygen consumption and viability is to be noted. A similar resistance was obtained for pellets of *Mycrothecium verrucaria*.

TABLE IV
EFFECTIVENESS OF VARIOUS TOXICANTS IN DECREASING OXYGEN CONSUMPTION AND
REDUCING VIABILITY OF MYCELIAL PELLETS

| Toxicant | ED ₅₀ values in µg. of toxicant in applied solution per mg. of pellets | | | | | |
|-----------------|--|-----------|----------------------------|-----------|----------------------------|-----------|
| | <i>Alternaria oleracea</i> | | <i>Aspergillus niger</i> | | <i>Monilinia fruticola</i> | |
| | Oxygen consump- tion | Viability | Oxygen consump- tion | Viability | Oxygen consump- tion | Viability |
| Silver | 0.27 | 0.25 | 0.27 | 0.25 | 0.06 | 0.16 |
| Copper | 0.52 | >158 | >61 | >61 | 1.3 | 6.0 |
| Mercury | 0.40 | 0.45 | 0.17 | 0.31 | 0.08 | 0.12 |
| Cadmium | >96 | >96 | >138 | >138 | >114 | >114 |
| Zinc | >400 | >400 | 600 | >400 | >169 | >169 |
| Cerium | >90 | >90 | >73 | >73 | >40 | >40 |
| Sodium arsenate | >296 | >296 | >188 | >188 | 120 | >600 |
| Malachite green | 8.6 | 70 | 8.0 | 100 | 0.50 | 0.10 |
| Cycloheximide | >67 | >67 | >24 | >24 | >59 | 8.5 |

Tests were run on the effects of silver and mercury on the oxygen consumption and viability of pellets of *Alternaria oleracea* and *Aspergillus niger* with and without 1 per cent sucrose. While the sucrose increased the oxygen consumption slightly as shown above, the effect in the presence of toxicants was proportional so that ED₅₀ values remained essentially the same for oxygen consumption and viability regardless of whether or not sucrose was present.

Comparative sensitivity of pellets and spores. Comparisons of the relative sensitivity of pellets and spores on a fresh weight basis as measured by

TABLE V
COMPARISON OF ED₅₀ VALUES OF PELLETS AND SPORES ON A FRESH WEIGHT BASIS
FOR VARIOUS TOXICANTS AND FUNGI BASED ON OXYGEN CONSUMPTION
AND VIABILITY

| Toxicant | Ratio of ED ₅₀ values for pellets/spores | | | | | |
|-----------------|---|-----------|----------------------------|-----------|----------------------------|-----------|
| | <i>Alternaria oleracea</i> | | <i>Aspergillus niger</i> | | <i>Monilinia fruticola</i> | |
| | Oxygen consump- tion | Viability | Oxygen consump- tion | Viability | Oxygen consump- tion | Viability |
| Silver | 0.42 | 0.60 | 0.02 | 0.19 | 0.20 | 1.14 |
| Copper | 0.03 | >31 | —* | >31 | 0.61 | 3.64 |
| Mercury | 0.03 | 0.05 | 0.01 | 0.19 | 0.05 | 0.10 |
| Cadmium | >0.04 | >3.0 | — | >0.09 | >100 | >200 |
| Zinc | >0.10 | >3.3 | <0.09 | — | >0.36 | >3.1 |
| Cerium | — | >8.2 | — | — | >1.0 | >7.7 |
| Sodium arsenate | — | >160 | — | — | 6.0 | >430 |
| Malachite green | 0.05 | 3.5 | <0.12 | <1.52 | 0.04 | 0.04 |
| Cycloheximide | — | >2.2 | — | >0.20 | >2.3 | 1.0 |

* Dashes indicate ED₅₀ values for both pellets and spores were indefinite.

oxygen consumption and germination or viability at the ED₅₀ level have been made on the data in Table IV and that of the earlier work on spores (9, Tables VI and VII). The ratios ED₅₀ for pellets/ED₅₀ for spores are given in Table V. Values less than 1 indicate that the pellets are more sensitive and values greater than 1 indicate that the spores are more sensitive. While there are various exceptions, usually reduction in oxygen consumption is a more sensitive response to toxicants for pellets and viability is a more sensitive response for spores.

Time of contact. The effects of suspending pellets in solutions of silver or mercury for 10 minutes, 1 hour, and 6 hours upon subsequent growth are summarized in Table VI. Data were also obtained for cadmium, zinc,

TABLE VI
EFFECT ON VIABILITY OF EXPOSING MYCELIAL PELLETS FOR VARYING TIME PERIODS TO SILVER OR MERCURY IONS

| Fungus | ED ₅₀ values in μ g. of toxicant in applied solution per mg. of pellets | | | | | |
|----------------------------|--|-------|--------|---------|-------|--------|
| | Silver | | | Mercury | | |
| | 10 min. | 1 hr. | 6 hrs. | 10 min. | 1 hr. | 6 hrs. |
| <i>Alternaria oleracea</i> | 15 | 3 | 1.2 | 24 | 8 | 3 |
| <i>Aspergillus niger</i> | 45 | 13 | 3 | 17 | 5 | 3 |
| <i>Monilinia fruticola</i> | 7 | 4 | 1.3 | >13 | 13 | 6 |

and cerium but in all instances external concentrations ranging from 5000 to 40,000 μ g. per mg. of pellets were without deleterious effect upon subsequent growth. In Table VI the marked effect of increasing time of exposure upon toxicity may be noted. Within the limits of these tests it may be observed that a 6-fold increase in time lowers the ED₅₀ value to one-third. Since this is a logarithmic relation it follows that toxicity doubles for each 3-fold increase in time of exposure. It will be noted in comparison with Table IV, that a 6-hour suspension in a given toxicant in a centrifuge tube has much less toxic effect on subsequent growth than does a 5-hour exposure in the microrespirometer. Presumably this difference is due to the higher temperature, 30° C., and constant shaking in the respirometer as compared to the lower temperature and occasional stirring when exposures are made in test tubes.

FUNGICIDE UPTAKE STUDIES

The results of typical studies to determine the uptake of silver by mycelial pellets of *Alternaria oleracea* and *Aspergillus niger* employing radioisotope techniques are shown in Tables VII and VIII. It will be noted that the uptake of silver is much slower and much less extensive than that previously reported for spores which can take up lethal doses within 30

TABLE VII
UPTAKE OF SILVER BY MYCELIAL PELLETS OF *ALTERNARIA OLERACEA* AND
ITS EFFECT ON VIABILITY

| Ag added, μg./g. | Cumulative uptake, μg./g. after time intervals in minutes | | | | | | Per cent viable after 1372 min. |
|---------------------|--|------|-----|-----|------|------|---------------------------------------|
| | 2.5 | 12.5 | 32 | 123 | 1122 | 1372 | |
| 26 | 4 | 5 | 9 | 11 | 13 | 15 | 100 |
| 109 | 20 | 43 | 56 | 62 | 70 | 75 | 100 |
| 628 | 213 | 292 | 355 | 392 | 430 | 444 | 0 |
| 3050 | 290 | 678 | 901 | 989 | 1060 | 1120 | 0 |

seconds. In Table VII the uptake reported was continuing up to at least 1372 minutes and at the lowest dose only about 60 per cent of that available had been taken up, while at the highest dose but little over a third had been taken up. After 1372 minutes the ED₅₀ value was approximately 200 μg./g. The ED₅₀ value for spores is 365 μg./g. (11). A somewhat similar uptake of silver by pellets of *Aspergillus niger* may be seen in the data of Table VIII except that higher dosages are required for comparable toxicity. The ED₅₀ value after 215 minutes is 1500 μg./g. but after 1255 minutes this is reduced to 500 μg./g., thus demonstrating the effect of time on the toxicity toward pellets. The previously reported ED₅₀ value for spores is 560 μg./g. (11).

It was considered possible that the slow rate of uptake by the mycelial pellets could be due to the slow rate of penetration through the mass of the pellet. A comparison was made of the silver uptake by large and small mycelial pellets of *Aspergillus niger*. The large pellets averaged 9 mg. fresh weight and the small 2 mg. There was no appreciable difference in uptake of the two sizes. In other tests pellets of *A. niger* and *Alternaria oleracea* were comminuted in a Waring Blendor or VirTis '45' Homogenizer for four different lengths of time. For the shortest period of 3 seconds the pellets were broken apart and for the longest period of 12 minutes the

TABLE VIII
UPTAKE OF SILVER BY MYCELIAL PELLETS OF *ASPERGILLUS NIGER* AND
ITS EFFECT ON VIABILITY

| Ag added, μg./g. | Cumulative uptake, μg./g. after time intervals in minutes | | | | | | Per cent viable after min. | |
|---------------------|--|------|------|------|------|------|-------------------------------|------|
| | 5 | 10 | 40 | 115 | 215 | 1255 | 215 | 1255 |
| 19 | 6 | 10 | 12 | 14 | 15 | 15 | 100 | 100 |
| 82 | 29 | 41 | 50 | 59 | 62 | 54 | 100 | 100 |
| 459 | 87 | 147 | 212 | 260 | 304 | 333 | 100 | 100 |
| 2,050 | 504 | 520 | 783 | 867 | 922 | 952 | 70 | 0 |
| 12,400 | 1750 | 2000 | 2660 | 1990 | 2380 | 2330 | 30 | 0 |
| 63,900 | 5200 | 7660 | 8650 | 8320 | 7080 | 7010 | 0 | 0 |

TABLE IX
UPTAKE OF SILVER BY WHOLE AND COMMINUTED PELLETS OF *ASPERGILLUS NIGER*
AND *ALTERNARIA OLERACEA*

| Fungus | Pellet treatment | Ag added, $\mu\text{g./g.}$ | Cumulative uptake, $\mu\text{g./g.}$ after min. | | |
|----------------------------|------------------|-----------------------------|---|------|------|
| | | | 5 | 45 | 1095 |
| <i>Aspergillus niger</i> | Whole | 24.5 | 5.9 | 8.2 | 10.5 |
| | | 21.3 | 18.0 | 19.0 | 20.0 |
| <i>Alternaria oleracea</i> | Whole | 19.0 | 8.0 | 8.2 | 8.2 |
| | | 17.2 | 6.8 | 6.9 | 7.9 |

mycelium was reduced to pieces varying from about 50 to 500 μ for *A. niger* and about 10 to 50 μ for *A. oleracea*. There was no difference in the amount of silver taken up by the different lots of the same fungus. The mean results of the comminuted pellets are compared to the whole pellets in Table IX. The uptake of silver by comminuted pellets of *Aspergillus niger* was increased 3-fold over the whole pellets after 5 minutes, but after 1100 minutes the difference was only 2-fold. There was, however, no difference with the pellets of *Alternaria oleracea*. Comminuted pellets will grow readily but under conditions similar to the above the oxygen consumption of comminuted pellets of *A. niger* was reduced to one-sixtieth.

TABLE X
UPTAKE OF DICHLONE BY MYCELIAL PELLETS OF *ALTERNARIA OLERACEA* AND
ASPERGILLUS NIGER AND ITS EFFECT ON VIABILITY

| <i>Alternaria oleracea</i> | | | | | | |
|---|---|-----------|--------|-----------|--------|-----------|
| Dichlone added, $\mu\text{g./g.}$ pellets | Cumulative uptake in $\mu\text{g./g.}$ and % viable after various time intervals in minutes | | | | | |
| | 60 | | 210 | | 1230 | |
| | Uptake | Viability | Uptake | Viability | Uptake | Viability |
| 37 | 32 | 90 | 35 | 100 | 36 | 100 |
| 70 | 44 | 90 | 58 | 100 | 63 | 100 |
| 163 | 75 | 100 | 103 | 90 | 112 | 70 |
| 362 | 189 | 80 | 193 | 60 | 209 | 30 |
| 668 | 284 | 50 | 377 | 80 | 414 | 70 |
| 1020 | 312 | 40 | 443 | 40 | 662 | 20 |
| 3620 | 778 | 70 | 1450 | 30 | 1490 | 0 |
| 4730 | 1720 | 0 | 2350 | 10 | — | — |
| <i>Aspergillus niger</i> | | | | | | |
| 23 | 16 | 100 | 20 | 100 | 20 | 100 |
| 45 | 29 | 100 | 34 | 100 | 37 | 100 |
| 81 | 32 | 100 | 47 | 100 | 54 | 100 |
| 169 | 63 | 100 | 82 | 100 | 93 | 100 |
| 321 | 85 | 100 | 139 | 100 | 165 | 100 |
| 921 | 346 | 90 | 376 | 100 | 394 | 100 |
| 2200 | 937 | 100 | 960 | 100 | 1030 | 100 |
| 5210 | 1700 | 100 | 1940 | 100 | 2160 | 100 |

The uptake of dichlone by pellets of *Alternaria oleracea* and *Aspergillus niger* is shown in Table X. The rate of uptake for the two species respectively was similar to that for silver. However, dichlone was toxic to *A. oleracea* with ED₅₀ values ranging from 700 to 300 µg./g. depending on time. An ED₅₀ value of 1150 µg./g. was obtained for the spores (9). With *A. niger* pellets there was no evidence of toxicity even after an uptake in excess of 2000 µg./g. A recent determination of the ED₅₀ for *A. niger* spores indicates a value of 10,000 µg./g.

It was also found that cerium was taken up by pellets of *Alternaria oleracea* in a manner comparable to that for silver. There was no toxic effect with uptake in amounts up to 510 µg./g. With *Aspergillus niger* there was no measurable uptake of cerium by the mycelial pellets despite the marked affinity of the spores for cerium (13).

Pellets of *Aspergillus niger* and *Monilinia fruticola* were exposed to radioactive zinc over a wide range of doses but the amounts taken up even after more than 40 hours' exposure were insignificant. As would be expected there was no effect on viability. In contrast the spores of these species take up appreciable amounts of zinc though very slowly (10).

A comparison of the ED₅₀ values for spores and for mycelial pellets of the same fungus when comparable data for the same toxicant are available indicates that they are of the same order of magnitude. That is, the limited data show the spores and mycelium on a fresh weight basis do not differ significantly in sensitivity to toxicants actually taken up.

DISCUSSION

There is relatively little knowledge concerning the respiration of filamentous fungi, according to Darby and Goddard (3). These authors studied the respiration of mycelium of *Myrothecium verrucaria*. Comminuting mycelial mats markedly reduced the oxygen consumption as has been found with *Aspergillus niger* in the present study. The rate of respiration was found to vary greatly with age of culture and was at a maximum for pellets from shaken cultures 20 to 30 hours old. Also, unless the cultures were starved, added substrates were not readily respired. These two factors probably explain the lack of marked oxygen consumption in the presence of sugar in the present experiments which were performed with older pellets taken directly from nutrient cultures.

In their studies on the antifungal action of sulfur compounds Klöpping (5), and van der Kerk and Klöpping (15) determined the inhibitory concentrations for roll cultures inoculated with spores of *Aspergillus niger* and *Penicillium italicum* and two other species, for shaken cultures of mycelial pellets of the above named species and for reduction in oxygen consumption by the pellets. There was fair agreement in inhibitory concentrations for the first two responses. However, it was found that compounds of higher specificity, i.e. active against certain fungi but not against others, which

were markedly effective in inhibiting growth, were much less active in reducing oxygen consumption. Whereas for compounds of low specificity, for which the toxicity to different fungi is similar, growth and oxygen consumption was inhibited in the same concentration range. These latter compounds were assumed to be more or less general cell poisons. In the present studies with pellets and previous ones with spores (9) cycloheximide is a compound of high specificity and its effectiveness would agree with that found by the above workers. However, the differential action of the nonspecific compounds in the present and previous studies shows a lack of accord with the above findings. But it should be pointed out that these authors (5, 15) used a far higher ratio of fungicide to spores than to mycelium.

Horsfall and Rich (4) have reported briefly on the differential action of compounds on spore germination and growth. A number of compounds inhibited hyphal growth of *Monilinia fructicola* but did not affect spore germination. It is pointed out that the former response necessitates cell division.

Recently Walker (16) has studied the effect of nine organic fungicides on *Myrothecium verrucaria* as measured by the reduction of spore germination in fungistatic tests and the reduction of respiration of spores and of mycelial pellets. The spore respiration was believed to be more sensitive than the germination. This is contrary to the earlier work of the present authors (9); however, here the spore germination tests were fungicidal since the spores were removed from exposure to the toxicants in the micro-respirometer. Walker also concluded that on a dry weight basis mycelial respiration was generally less sensitive to toxicants than spore respiration. If the comparison were made on an equivalent fresh weight basis the mycelium would tend to be more sensitive as also indicated in Table V of the present studies.

Cochrane (2) using specifically labeled glucose has indicated that with some species, spore respiration differs quantitatively from mycelial respiration. Differences in sensitivity would thus be possible if the different pathways are not similarly affected by toxicants. However, with *Myrothecium verrucaria* no difference was observed in this respect between the respiration of the spores and the mycelium.

In another communication (8) the authors have recently shown that under anaerobic conditions both fungus conidia and mycelial pellets reduce sulfur to hydrogen sulfide with the formation of a molecular equivalent of additional carbon dioxide. On a fresh weight basis, however, the pellets are much less reactive. Under aerobic endogenous conditions sulfur increases the oxygen consumption of spores, while in the presence of glucose sulfur causes a marked decrease in oxygen consumption, followed by a rise and subsequent fall. In contrast pellets respond but little to sulfur either with or without glucose substrate.

It is commonly recognized that with the exception of lime sulfur and

the organic mercurials none of the established foliage fungicides such as Bordeaux mixture, the fixed coppers, the various dithiocarbamates, chloranil (tetrachloro-*p*-benzoquinone), dichlone (2,3-dichloro-1,4-naphthoquinone), or glyodin (2-heptadecyl-2-imidazoline acetate) are effective as eradicants. Many of these fungicides are known to be readily taken up by fungus spores responsible for initiating numerous diseases, and are good protectants. The fact that they fail as eradicants may be due in part to the difficulty and slowness with which mycelium will take up these toxicants. Even Karathane [2-(1-methylheptyl)-4,6-dinitrophenyl crotonate and isomers] which has given effective control of various powdery mildew diseases appears to act as a protectant rather than as an eradicant despite the exposed condition of the mycelium.

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EQUIMOLAR FORMATION OF CARBON DIOXIDE AND HYDROGEN SULFIDE WHEN FUNGUS TISSUE REDUCES SULFUR¹

S. E. A. MCCALLAN AND LAWRENCE P. MILLER

SUMMARY

The production of carbon dioxide and hydrogen sulfide by conidia of *Aspergillus niger*, *Monilinia fruticola* and *Neurospora sitophila*, cells of *Saccharomyces cerevisiae* and mycelial pellets of *A. niger* and *Alternaria oleracea* was determined under anaerobic conditions in the presence of colloidal sulfur. One mole of additional carbon dioxide, over and above the controls, was produced for every mole of hydrogen sulfide evolved by each fungus.

Under aerobic conditions, the oxygen consumption by the conidia was increased upon the addition of colloidal sulfur, but 12 hours later the rate had fallen to that of the controls. However, with a glucose substrate the addition of sulfur induced a marked fall in the oxygen consumption, followed by a marked rise and subsequent fall, so that at the end of 12 hours the total oxygen consumption was equivalent for the sulfur and control series. Mycelial pellets were much less responsive to sulfur and to glucose.

INTRODUCTION

Earlier studies were made on the role of hydrogen sulfide in the toxic action of sulfur (5) and on the effect of various fungitoxicants, including sulfur, on the oxygen consumption and germination of fungus spores (4). These investigations have been extended to include quantitative determinations of the amount of additional carbon dioxide which it has been found is produced when sulfur is reduced to hydrogen sulfide under anaerobic conditions. Also included are further tests on the effect of sulfur on oxygen consumption. Fungus spores, pellets, and yeast cells were studied employing sulfur in a colloidal form.

MATERIALS AND METHODS

Fungus conidia were obtained from cultures of *Aspergillus niger* von Tiegh, *Monilinia fruticola* (Wint.) Honey, and *Neurospora sitophila* (Mont.) Shear & Dodge as previously reported (4). Fungus pellets of *Aspergillus niger* and of *Alternaria oleracea* Milbraith were also employed. These were cultured as described in a prior communication (3). Finally cells of the commercial baker's yeast,² *Saccharomyces cerevisiae* Hansen,

¹ These investigations were conducted in cooperation with the United States Atomic Energy Commission, Contract AT(30-1)-788. A preliminary report was presented before the American Phytopathological Society, Atlanta, Georgia, December 1955 (2).

² Fleischmann's Active Dry Yeast for Baking.

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were also used. The amounts of fresh weight of tissue used for each determination depended on its activity. The quantities used were approximately as follows: conidia of *M. fructicola*, 13 to 25 mg.; *N. sitophila*, 18 to 30 mg.; *A. niger*, 23 to 54 mg.; pellets of *Alternaria oleracea*, 80 to 200 mg.; and of *Aspergillus niger*, 200 to 400 mg. Ten mg. of the dry yeast preparation were used.

Reagent forms of sulfur or wettable sulfur fungicides were unsuitable for these studies since the reaction is too slow and excessive amounts are required (4). Accordingly the colloidal sulfur used previously (5) was employed. This colloidal sulfur was prepared by dissolving 100 mg. of reagent sulfur in 200 ml. acetone. An amount of the solution added to an equal amount of water produced a colloidal milky suspension. The suspension was then evaporated under partial vacuum in a hot water bath to about one-sixth of its original volume in order to drive off all the acetone.

Standard microrespirometer techniques (8) were employed according to the details described earlier (4). The temperature was 30° C. and duration of tests was 5 hours (anaerobic) or 12 hours or more (aerobic) following the addition of 0.2 ml. of colloidal sulfur (about 0.2 mg. sulfur) from the side arm. In the anaerobic tests the apparatus was first freed of oxygen by passing nitrogen through for 45 minutes. No added substrate was used in the anaerobic tests, while for the aerobic series, 1 per cent glucose was used. The volume of liquid and fungus tissue was 2.0 ml. per flask.

The carbon dioxide and hydrogen sulfide were determined by the Direct Method of Warburg (8). This involved one set of flasks without sulfur and without an absorber to give the basal carbon dioxide production. A second set of flasks containing sulfur but no absorber gave the total combined carbon dioxide and hydrogen sulfide produced. Finally the third set containing sulfur and a hydrogen sulfide absorber, gave the total carbon dioxide. The hydrogen sulfide was determined by the difference between the second and third set of flasks, while any additional carbon dioxide produced as a result of the presence of the sulfur was given by the difference between the third and first set of flasks. Each variable was usually carried in triplicate for a given test. Following some experimentation, zinc acetate was selected as the hydrogen sulfide absorber and this was used at a concentration of 2 per cent. Folded strips of filter paper were added to the 0.2 or 0.3 ml. quantity of zinc acetate. A considerable number of comparisons were made in which the zinc acetate was placed in the center well only or in the center well and also the second side arm. When the zinc acetate absorber was placed in the center well only not all the hydrogen sulfide was absorbed as in general only about two-thirds as much hydrogen sulfide was obtained as when the additional absorber in the side arm was also used. Accordingly absorbers in both center well and side arm were used in all the tests reported. As a further check on the hydrogen sulfide absorbed, at the end of the run, the absorbed solutions were recovered and the

zinc sulfide determined iodimetrically³ (5, p. 155). In general these results were found to agree closely with those of the manometric analyses and showed no consistent nor significant difference.

All results of carbon dioxide or hydrogen sulfide production or oxygen consumption were calculated as microliters of gas per mg. of fungus tissue (fresh weight) per hour or per 5 hours. The yeast data are based on the weight of the commercial preparation.

TABLE I

ADDITIONAL CO₂ PRODUCED AS SULFUR IS REDUCED TO HYDROGEN SULFIDE UNDER ANAEROBIC CONDITIONS BY CONIDIA OF THREE FUNGI, AND BY YEAST CELLS IN FOUR TESTS EACH

| <i>Aspergillus niger</i> | | | | | |
|---------------------------------|----------------------------|-------|------|------|------|
| Gas produced | μl. gas per mg. in 5 hours | | | | Mean |
| Basal CO ₂ | 1.41 | 1.57 | 1.67 | 1.20 | 1.46 |
| Hydrogen sulfide | 2.88 | 4.21 | 1.44 | 3.24 | 2.94 |
| Additional CO ₂ | 3.98 | 4.48 | 1.22 | 3.06 | 3.19 |
| <i>Monilinia fruticola</i> | | | | | |
| Basal CO ₂ | 0.89 | 0.65 | 0.72 | 0.91 | 0.79 |
| Hydrogen sulfide | 2.15 | 5.45 | 7.18 | 8.30 | 5.77 |
| Additional CO ₂ | 2.75 | 5.06 | 5.41 | 5.70 | 4.73 |
| <i>Neurospora sitophila</i> | | | | | |
| Basal CO ₂ | 1.38 | 1.03 | 1.70 | 1.39 | 1.38 |
| Hydrogen sulfide | 3.05 | 3.22 | 5.52 | 4.63 | 4.10 |
| Additional CO ₂ | 2.59 | 3.17 | 4.23 | 3.82 | 3.45 |
| <i>Saccharomyces cerevisiae</i> | | | | | |
| Basal CO ₂ | 10.15 | 8.55 | 9.51 | 9.93 | 9.54 |
| Hydrogen sulfide | 1.41 | 10.20 | 7.29 | 7.32 | 6.58 |
| Additional CO ₂ | 2.02 | 10.68 | 4.06 | 3.85 | 5.15 |

RESULTS

PRODUCTION OF ADDITIONAL CARBON DIOXIDE AS SULFUR
IS REDUCED TO HYDROGEN SULFIDE

The results of four tests with the conidia of three fungi and with yeast cells are reported in Table I for the production of basal carbon dioxide, hydrogen sulfide, and additional carbon dioxide. Similar results were obtained for the mycelial pellets of *Alternaria oleracea* and *Aspergillus niger*, but, since they are more variable, seven tests were run. These are reported in Table II. It will be seen in Table I that the rate of production of carbon dioxide in the absence of sulfur is relatively constant in the different tests

³ The authors are indebted to Mrs. Joan Szabo-Pelsöczy for making the iodimetric analyses.

TABLE II

ADDITIONAL CO₂ PRODUCED AS SULFUR IS REDUCED TO HYDROGEN SULFIDE UNDER ANAEROBIC CONDITIONS BY MYCELIAL PELLETS OF TWO FUNGI IN SEVEN TESTS EACH

| <i>Alternaria oleracea</i> | | | | | | | | |
|----------------------------|---------------------------------|-------|-------|-------|-------|-------|-------|-------|
| Gas produced | μ l. gas per mg. in 5 hours | | | | | | | Mean |
| Basal CO ₂ | 1.39 | 1.93 | 1.55 | 1.53 | 2.09 | 2.36 | 1.18 | 1.72 |
| Hydrogen sulfide | 0.96 | 0.30 | 0.27 | 0.09 | 0.27 | 0.51 | 0.24 | 0.38 |
| Additional CO ₂ | 0.50 | 0.37 | 0.15 | 0.09 | 0.13 | 0.26 | 0.28 | 0.26 |
| <i>Aspergillus niger</i> | | | | | | | | |
| Basal CO ₂ | 0.702 | 0.426 | 0.329 | 0.336 | 0.313 | 0.077 | 0.157 | 0.334 |
| Hydrogen sulfide | 0.059 | 0.040 | 0.056 | 0.040 | 0.042 | 0.028 | 0.027 | 0.041 |
| Additional CO ₂ | 0.075 | 0.082 | 0.064 | 0.039 | 0.032 | 0.036 | 0.023 | 0.050 |

for the conidia and yeast cells. However, the species differ markedly (the L.S.D. at 5 per cent for the means is 0.66) and the yeast cells have the highest rate as might be expected. The mycelial pellets (Table II) show greater variability and the rate for *Aspergillus niger* is low and only about one-fourth that for the corresponding conidia.

The hydrogen sulfide values given in Tables I and II are based on the mean of the manometric and iodimetric determinations. It will be seen that these values are more variable than those for the basal carbon dioxide production. This is probably due to differences in the physical state of the colloidal sulfur and perhaps also to variability in physiological activity of the different lots of spores, cells, and mycelial pellets. The spores and yeast cells are active producers of hydrogen sulfide as shown earlier (5); however, the differences between species are less under the present conditions. The mycelial pellets are much less active than the conidia and yeast cells in reducing sulfur and with *Aspergillus niger* there is a 70-fold difference between conidia and mycelium. The lower reactivity of the mycelial pellets in contrast to the spores is in accord with the previous studies (3).

The additional carbon dioxide produced upon the introduction of sulfur will be seen to follow fairly closely the corresponding values for hydrogen sulfide as given in Tables I and II. A detailed comparison has been made of the individual additional carbon dioxide and the associated hydrogen sulfide values including both manometric and iodimetric determinations of the latter. These data expressed as the ratio CO₂/H₂S have been summarized in Table III together with the standard deviations as obtained from an analysis of variance (7). It will be seen that none of the fungi differ significantly from one another, nor does the ratio differ significantly from unity. That is, for every mole of hydrogen sulfide produced there is an equivalent mole of additional carbon dioxide evolved. It seems of particular significance that this ratio of unity should hold for the conidia of all

TABLE III

MEAN RATIOS AND STANDARD DEVIATIONS OF ADDITIONAL CO₂ PRODUCTION TO H₂S
PRODUCTION IN THE PRESENCE OF SULFUR UNDER ANAEROBIC CONDITIONS
FOR VARIOUS FUNGI

| Fungus | Form | Mean CO ₂ /H ₂ S | Standard deviation |
|---------------------------------|----------|--|--------------------|
| <i>Aspergillus niger</i> | Conidia | 1.07 | 0.11 |
| <i>Monilinia fructicola</i> | Conidia | 0.93 | 0.22 |
| <i>Neurospora sitophila</i> | Conidia | 0.88 | 0.18 |
| <i>Saccharomyces cerevisiae</i> | Cells | 0.89 | 0.10 |
| <i>Alternaria oleracea</i> | Mycelium | 0.87 | 0.55 |
| <i>Aspergillus niger</i> | Mycelium | 1.27 | 0.37 |

the species studied as well as the mycelial pellets and the yeast cells.

The ratio of additional carbon dioxide produced to hydrogen sulfide produced may be shown graphically with the passage of time. In Figure 1 there is plotted the rate of gas production per hour against elapsed time from the addition of sulfur for some individual experiments with conidia of *Aspergillus niger*, *Monilinia fructicola* and *Neurospora sitophila* and cells of *Saccharomyces cerevisiae*. For comparison the basal carbon dioxide production curves are also given. Of necessity these data are derived from the manometric measurements only. It will be seen that the basal carbon dioxide curves for the conidia tend to change but little and maintain a more or less constant rate. On the other hand, the additional carbon dioxide and hydrogen sulfide curves tend to fall off rapidly especially for *M. fructicola* and *N. sitophila*. Ideally for a ratio of unity the additional carbon dioxide and hydrogen sulfide curves would be expected to coincide. These individual curves more or less approach this condition.

EFFECT OF SULFUR ON OXYGEN CONSUMPTION

In the earlier studies on oxygen consumption (4) sulfur had been included among the fungitoxicants. However, a commercial wettable sulfur had been used and because of its slow reactivity such large quantities were needed that there was a question as to the meaning of the results obtained. It therefore was decided to run tests with colloidal sulfur. But first the respiratory quotients were obtained for the conidia and pellets under the

TABLE IV

MEAN R.Q. VALUES AND STANDARD DEVIATIONS FOR CONIDIA AND MYCELIAL PELLETS
OF SEVERAL FUNGI DETERMINED ENDOGENOUSLY AND IN PRESENCE OF
GLUCOSE SUBSTRATE

| Fungus | Endogenous | Glucose substrate |
|--------------------------------------|--------------|-------------------|
| <i>Aspergillus niger</i> —conidia | 0.82 ± 0.11 | 1.04 ± 0.065 |
| <i>Monilinia fructicola</i> —conidia | 0.72 ± 0.016 | 1.19 ± 0.002 |
| <i>Neurospora sitophila</i> —conidia | 0.73 ± 0.004 | 1.18 ± 0.009 |
| <i>Alternaria oleracea</i> —pellets | 0.95 ± 0.11 | 1.17 ± 0.063 |
| <i>Aspergillus niger</i> —pellets | 0.94 ± 0.11 | 1.37 ± 0.22 |

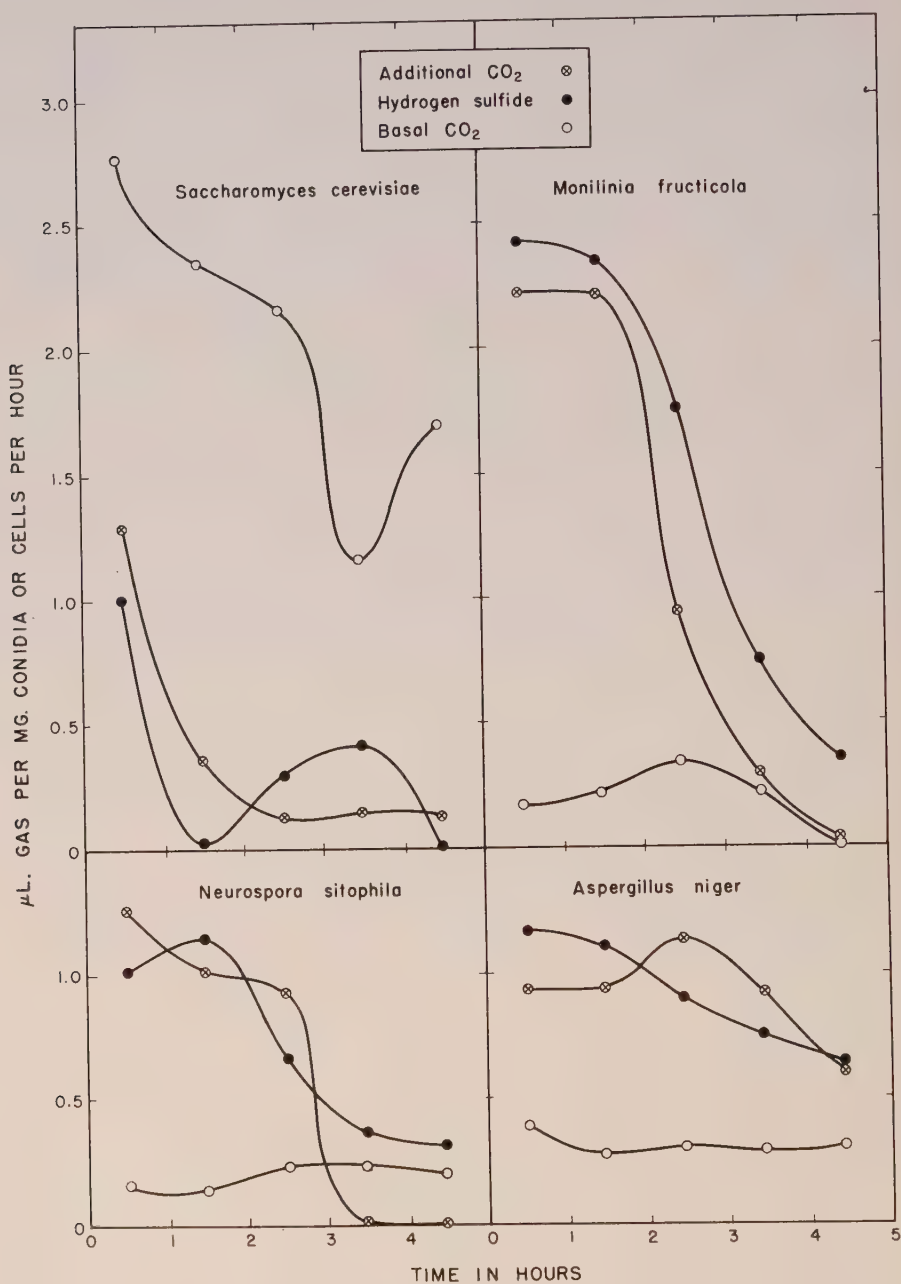


FIGURE 1. Rate of production of additional carbon dioxide, hydrogen sulfide, and basal carbon dioxide by conidia of three fungi and by yeast cells in contact with sulfur under anaerobic conditions. Single test each.

conditions of the tests employed. The mean results of two tests with the conidia and three with the pellets are shown in Table IV. It will be seen that the endogenous values for the conidia are similar for the three species and that the value for *N. sitophila* agrees closely with that of 0.74 reported by Owens (6). Such values are considered characteristic of fat metabolism. The endogenous values for the pellets are significantly higher than those of the conidia indicating a different metabolism. In the presence of glucose the R.Q. values for both conidia and pellets do not differ significantly from unity, the theoretical value for complete oxidation of the glucose.

The results on the effect of sulfur on oxygen taken up by the above fungi are recorded in Figure 2 for periods of 12 hours or more based on the mean results of two tests each. The oxygen consumption in the absence of glucose does not differ appreciably for the three species and from the beginning falls asymptotically. However, immediately upon the addition of sulfur there is a marked increase in the oxygen consumption with a maximum after the second to fourth hour. By the end of 12 hours the rate has fallen almost to that of controls. Thus during the total run the introduction of the sulfur resulted in an approximate doubling of the oxygen taken up. The actual ratios are *Neurospora sitophila* 2.1, *Monilinia fructicola* 2.1, and *Aspergillus niger* 1.9.

In the presence of the glucose substrate the oxygen consumption is markedly increased for *Neurospora sitophila* and *Monilinia fructicola* with little change for *Aspergillus niger* as noted previously (4). The addition of the sulfur brings about an immediate and pronounced fall in the rate of oxygen consumption; this in turn is followed by a rise with a maximum some 3 to 9 hours after the addition of the sulfur. By the end of 12 to 15 hours the rate has returned to that of the controls. The net result is an oxygen consumption slightly more than or equal to that of the controls. The ratios are *Neurospora sitophila* 1.0, *Monilinia fructicola* 1.1, and *Aspergillus niger* 1.3. The results with *N. sitophila* and *A. niger* are in general agreement with those previously reported (4) except for the difference in amount of sulfur involved. With *M. fructicola* the stimulation was observed earlier but the present depression was not noted.

Similar studies with the mycelial pellets of *Alternaria oleracea* and *Aspergillus niger* showed that they were much less responsive to the addition of sulfur. With both species the initial control rate of oxygen consumption was about 0.5 to 1.0 μ l. per hour per mg. of fresh weight. The presence of the glucose substrate gave little or no increase in the rate. The addition of sulfur to the pellets of *A. oleracea* in a glucose medium, as shown in Figure 2, resulted in an immediate fall in the rate of oxygen uptake, but after 6 hours the rate had returned to that of the controls. The presence of the glucose made no appreciable difference in the rate of fall and recovery. The addition of sulfur to the pellets of *A. niger* had relatively little effect on oxygen consumption either with or without glucose substrate.

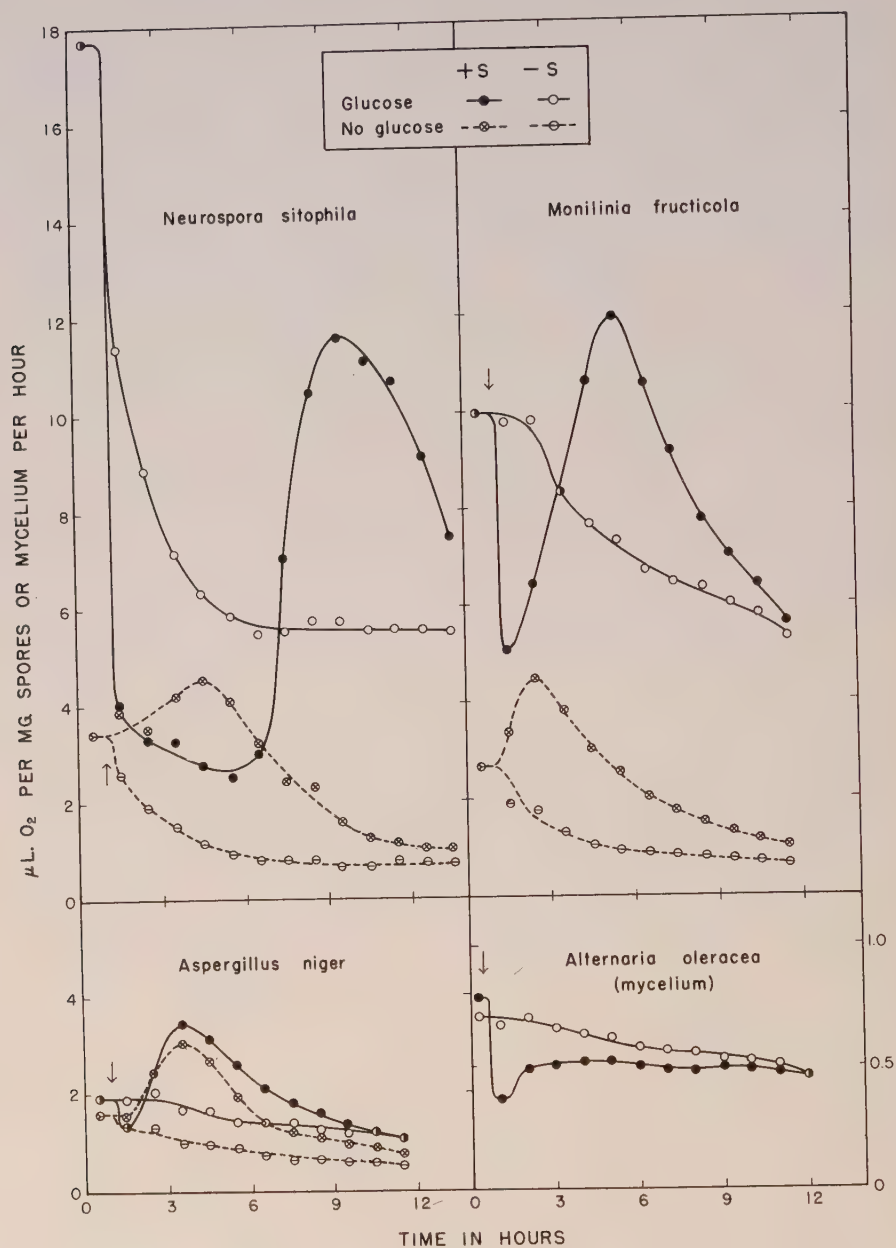


FIGURE 2. Rate of oxygen consumption by conidia of three species of fungi and by mycelial pellets of *Alternaria oleracea* as influenced by sulfur with and without glucose under aerobic conditions. Mean of two tests each. Sulfur added at point indicated by arrow. Note oxygen scale for *A. oleracea* is magnified 5 \times .

In an earlier paper (3) the change in coloration was described for the *Alternaria oleracea* pellets from light greyish-white to olive-brown or even black. This change takes place fairly rapidly when the pellets are several days old. It was noted that control pellets would change color while in the respirometer. However, the presence of the colloidal sulfur prevented the change in pigmentation.

DISCUSSION

The fact that a molecule of carbon dioxide is formed concomitant with reduction of sulfur to hydrogen sulfide by the fungus conidia, mycelium and yeast cells gives support to previous conclusions (5) that the hydrogen sulfide is probably not derived from a simple reaction between the sulfur and sulfhydryl compounds in the cells. As pointed out earlier, if sulfhydryl compounds are involved, a mechanism must be available for the ready reduction of the disulfide formed in the reaction with sulfur, since it is unlikely that the spores or other cells contain enough sulfhydryl compounds to account for the very large amounts of hydrogen sulfide which can be produced. Should carbon dioxide be formed in connection with the reduction of disulfides then the results of the present experiments could be reconciled with the above mechanism.

In these studies on the formation of additional carbon dioxide it has been assumed that the carbon dioxide formation from endogenous substrates under anaerobic conditions is not affected as such by the addition of elemental sulfur. If this be not true then entirely different interpretations will have to be given to the results obtained. The fact that the quantity of additional carbon dioxide produced is so closely related to the hydrogen sulfide evolved, adds credence to the supposition that there is a close relationship between the two. Cochrane (1) has recently shown that when glucose is added as substrate, carbon dioxide production from endogenous substrate may be suppressed, accelerated, or remain unaffected. The possibility of an effect of sulfur not directly connected with the formation of hydrogen sulfide can therefore not be ignored.

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INSECTICIDAL PROPERTIES AND CHARACTERISTICS OF 1-NAPHTHYL *N*-METHYLCARBAMATE¹

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AND HERBERT H. MOOREFIELD²

SUMMARY

1-Naphthyl *N*-methylcarbamate, representative of a new group of insecticidal carbamates, has been synthesized and tested as an insecticide. Laboratory evaluation is reported, and the results of preliminary field tests are presented. This new chemical, designated as Sevin, has exhibited a broad insecticidal activity and possesses a low order of mammalian toxicity. It is further characterized by moderate residual properties, some systemic effects, and a wide margin of safety from phytotoxicity. The primary mechanism of action as an insecticide appears to be that of an anticholinesterase.

INTRODUCTION

The cholinergic activity of synthetic *O*-phenyl carbamate drugs was demonstrated as early as 1926 (9). A recent review (6) has pointed out that subsequent research in this field has emphasized the groups of water-soluble quaternary salt or amine hydrochloride derivatives of certain carbamates. These compounds have been shown to be very effective inhibitors of insect cholinesterase, but of little or no value as insecticides (5).

Researches initiated in 1947 which culminated in a series of active heterocyclic carbamates were the first published records of the carbamate class being explored for their insecticidal properties (1, 2, 3). Later studies (5) extended this knowledge by showing that the anticholinesterase activity of certain nonpolar *O*-phenyl carbamates could be projected to exhibit high toxicity to insects.

Present studies have uncovered a new series of active polycyclic carbamates. One of these, 1-naphthyl *N*-methylcarbamate, possesses a desirable combination of a broad spectrum of insecticidal activity and a relatively low order of mammalian toxicity. This paper presents a preliminary report on certain chemical, physiological, and insecticidal properties of 1-naphthyl *N*-methylcarbamate. This chemical is currently being field tested and developed under the name of Sevin.

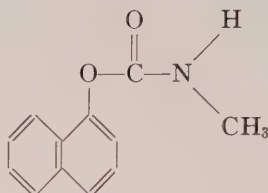
¹ This chemical has been field tested as "Experimental Insecticide 7744" identified as an aryl urethane.

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MATERIALS AND METHODS

Description of the chemical. 1-Naphthyl *N*-methylcarbamate is a white crystalline solid with a melting point of 145° C., vapor pressure of less than .005 mm. Hg at 26° C., and a density of 1.232 at 20/20° C. Technical samples are slightly colored. It is moderately soluble in organic solvents such as acetone, *N,N*-dimethylformamide, isophorone, and cyclohexanone. The solubility in water is less than 0.1 per cent. This chemical is stable to heat, light, and acid conditions but is hydrolyzed in highly alkaline media. Both technical material and field formulations have proved to be stable under normal storage conditions. The structural formula is:



Detailed methods of preparation and analytical procedures for determination of residues will be published elsewhere.

Laboratory testing procedures. The apparatus, conditions and general procedures employed in tests against bean aphid, *Aphis fabae* Scop.; two-spotted spider mite, *Tetranychus telarius* (L.); southern armyworm, *Prodenia eridania* (Cram.); and Mexican bean beetle, *Epilachna varivestis* Muls., have been described in detail (4). Test formulations were made by dissolving Sevin in acetone and Tergitol Dispersant NPX⁴ and diluting with water to give the desired concentration.

Aphid tests were performed in pots containing 6 to 10 nasturtium (*Tropaeolum majus* L.) plants. Standardized infestations of 100 to 150 adults and nymphs were accomplished by thinning out the plants. After spraying to a point of run-off (on a turntable apparatus), each pot was placed on its side in a Petri dish containing a piece of white, ruled paper, ringed with Tanglefoot to prevent escape of the test insects. Counts on the living and dead insects remaining on the plants and those which had fallen onto the paper were made 24 hours after treatment.

In the mite tests, pots containing a single bean, *Phaseolus vulgaris* L. var. Tendergreen, plant were similarly sprayed 24 hours after infestation with approximately 100 adult and nymphal mites. Mortality was determined by examination with a dissecting microscope 48 hours after treatment. Bean aphids and spider mites were used routinely in tests to determine systemic insecticidal action. Infested plants were first uprooted. The exposed roots were rinsed to remove soil and then immersed in aqueous

⁴ An alkyphenyl polyethylene glycol ether, Carbide and Carbon Chemicals Co.

formulations of insecticide. Records of control were taken 48 hours after starting the test.

Mexican bean beetle and southern armyworm evaluation tests were made on excised bean leaves. Two leaves which had been dipped in insecticide mixture and allowed to dry were placed on moistened filter paper in a covered Petri dish. Either third-instar bean beetle larvae or 8- to 9-day-old armyworm larvae were placed on the leaves. Percentage mortality was recorded after a 72-hour period.

House flies, *Musca domestica* L., were reared by standardized Peet-Grady procedures (8). For fly bait tests, 25 adults were placed under five-inch hemispherical wire cages. A wad of cotton, saturated with a sugar solution to which the insecticide had been added, was placed in each cage and served as the only source of food. Counts were taken after 24-hour exposure.

In all of the above tests the chemicals were applied in a series that would yield dosage-mortality lines with at least four reference points. A minimum of four replicate determinations have been performed in all laboratory evaluations and the LD₅₀ values (derived from log-probability plots) presented are an average of the results obtained. All figures have been corrected for natural mortality in the untreated controls.

Field tests. Extensive field trials have been conducted in 34 states under the supervision of State and Federal cooperators. The experiments, ranging in size from small plot tests to commercial acreage, were of conventional design and executed under controlled conditions. Equivalent untreated plots and comparative treatments with recommended proprietary insecticides were simultaneously assessed.

Sevin was evaluated in formulations of 25 and 50 per cent wettable powders, 24 per cent emulsifiable concentrates and 5 per cent dusts. The tests were performed at rates of one-half to two pounds of active ingredient per acre or applied at these doses contained in 100 gallons of water.

Details of the individual evaluations describing experimental designs, specific formulations, crops, insect infestations, and control effected will be released for publication at the discretion of the independent investigators.

EXPERIMENTAL RESULTS AND DISCUSSION

Laboratory insecticidal evaluation. The relative performance of Sevin and several common insecticides is presented in Table I. In these comparisons the carbamate was found to be highly effective on Mexican bean beetle, bean aphid, and southern armyworm. It was not toxic to mites and, by the method of application used in these experiments, it provided only slight control of house flies.

No systemic toxicity to the two-spotted mite on bean foliage was

TABLE I

COMPARATIVE PERFORMANCE OF 1-NAPHTHYL *N*-METHYLCARBAMATE AND OTHER
COMMON INSECTICIDES IN LABORATORY TESTS (FIGURES ARE LD₅₀
VALUES IN MG./100 ML.)

| Insecticide | Mexican bean beetle | Bean aphid | Southern armyworm | House fly bait test | Two-spotted spider mite |
|--------------|------------------------|---------------|----------------------|------------------------|----------------------------|
| Sevin | 0.8 | 2 | 10 | 20 | > 150 |
| Malathion | 10 | 3 | 20 | 3 | 2 |
| Methoxychlor | 375 | 9 | 35 | 6 | > 150 |
| DDT | > 125 | 6 | 5 | 2 | > 150 |
| Aldrin | 90 | 20 | 1.4 | 2 | > 150 |
| Toxaphene | 8 | 12 | 5 | 10 | 150 |

found, but a low level of systemic activity against bean aphids infesting nasturtium plants prompted further experimentation.

Systemic activity. In order to learn if Sevin would accumulate in plants through prolonged exposure in the soil, a dosage series of 5 per cent dust formulations were mechanically blended with soil samples. Tendergreen bean seeds were planted, and after 3 to 4 weeks, trifoliate leaves were presented to Mexican bean beetle larvae as previously described. At rates equivalent to 128 lbs. of chemical per acre, 80 per cent of the larvae were killed and little or no damage to the leaves was encountered from feeding. At 32 lbs. per acre, 25 per cent of the leaves were consumed by the larvae and 40 per cent control was obtained. At lower doses, the insects were unaffected.

The systemic test was further modified by dissolving the carbamate in acetone, emulsifying in water with Triton X-155⁵ (10:1 w/w) and incorporating it into the soil. Nasturtium and Tendergreen bean seeds were planted three days later. When the plants were 11 days old, bean aphid adults and two-spotted spider mites were placed on the foliage, and Mexican bean beetle and southern armyworm were allowed to feed on excised leaves. In these experiments, the beetle larvae showed 55 per cent mortality at rates of 40 lbs. of chemical per acre, whereas no effect was noticed on the other test insects.

Preliminary trials of testing this compound as a seed treatment have been carried out by coating bean seeds with a 50 per cent wettable powder slurry at 2 lbs. per 100 lbs. of seeds. Leaves from 14-day-old bean plants so treated gave complete control of exposed Mexican bean beetle and 50 per cent kill of southern armyworm larvae.

Field insecticidal evaluation. As the specific results of cooperative field projects generally become the property of the agency performing the tests, only selective reports indicative of promising control for major economic pests have been considered for this paper.

⁵ An alkyl aryl polyether alcohol, Rohm and Haas Co.

In every instance where Sevin has been evaluated as a control measure for the codling moth, *Carpocapsa pomonella* (L.), outstanding performance has been reported. Other fruit insects which have been efficiently controlled with this chemical include the red-banded leaf roller, *Argyrotaenia velutinana* (Wlk.); oriental fruit moth, *Grapholitha molesta* (Busck); rosy apple aphid, *Anuraphis roseus* Baker; and the grape leaf folder, *Desmia funeralis* (Hbn.).

Excellent field control of the Mexican bean beetle has been obtained in several states. A high degree of toxicity for other coleopterous and lepidopterous pests such as the Colorado potato beetle, *Leptinotarsa decemlineata* (Say); green June beetle, *Cotinis nitida* (L.); potato flea beetle, *Epitrix cucumeris* (Har.); corn earworm, *Heliothis zea* (Boddie); yellow-striped armyworm, *Prodenia ornithogalli* Guen.; as well as the potato leafhopper, *Empoasca fabae* (Harr.), have been recorded on numerous vegetable crops.

Field evaluation of Sevin against cotton insects has been delayed because of formulation difficulties. Initial plot experiments indicate satisfactory control of the cotton bollworm (corn earworm). Based on excellent laboratory results, good field performance is anticipated against the boll weevil, *Anthonomus grandis* Boh.; pink bollworm, *Pectinophora gossypiella* (Saund.); and cotton aphid, *Aphis gossypii* Glov.

Only limited activity has been noted against the house fly, mites (various species), ants and grasshoppers. Observations on control of scale insects has been variable. Activity has been reported against the citricola scale, *Coccus pseudomagnoliarum* (Kuw.), and California red scale, *Aonidiella aurantii* (Mask.); whereas, no reduction of the olive scale, *Parlatoria oleae* (Colvée) could be discerned.

Mechanism of insecticidal action. The mode of action of this new insecticide appears to be primarily that of an anticholinesterase. Efficient inhibition of an insect-derived cholinesterase can be demonstrated by *in vitro* evaluation. Measured by standard manometric techniques (7) a 3.5×10^{-7} molar concentration of 1-naphthyl *N*-methylcarbamate is required to effect 50 per cent inhibition of a fly brain cholinesterase brei.

Involvement of cholinesterase inhibition in the general insect toxicity syndrome produced by Sevin can also be demonstrated with experiments designed to correlate enzyme studies with *in vivo* symptoms caused by other carbamate insecticides (5). House flies, carefully treated with topical microdrops of the carbamate in acetone applied onto the thorax, were decapitated in the prostrate stage of poisoning. Enzyme assays made on equivalent homogenates of heads of treated and control flies exhibited a marked decrease in the cholinesterase activity of those preparations made from poisoned insects.

Plant tolerance studies. Laboratory phytotoxicity tests have been con-

ducted on several varieties of plants including beans, *Phaseolus vulgaris* L. var. Tendergreen; corn, *Zea Mays* L. var. Cornell M-4; tomato, *Lycopersicon esculentum* Mill. var. Bonny Best; cotton, *Gossypium hirsutum* L. var. Coker-100; and tobacco, *Nicotiana tabacum* L. var. Maryland Mammoth. No injury has been observed in these tests with Sevin sprayed on plants at recommended field dosages, i.e., ranging from one-half to two pounds per acre or the same rates applied in 100 gallons of water.

Under actual field conditions, no phytotoxicity has been reported with wettable powders. Slight injury has been observed with one- and two-pound gallon emulsifiable concentrates, but further laboratory investigations suggest that this was caused by certain emulsifier-solvent combinations.

By a soil drench method, under laboratory conditions, slight injury was evident on established tomato plants when the technical chemical was applied at 25 times the recommended rate of foliage application. At equivalent rates, germination of radish (*Raphanus sativus* L.) seeds was slightly retarded and severe reduction in germination of perennial rye (*Lolium perenne* L.) seeds was obtained.

Mammalian toxicity. Studies on the mammalian toxicology of Sevin have been performed.⁶ Results of acute toxicity tests indicate an oral LD₅₀ for rats in the range of 500 to 700 mg./kg. In rabbit skin penetration tests the LD₅₀ has been shown to be greater than 2000 mg./kg. This carbamate manifests a low degree of chronicity in repeated oral doses to rats and appears to produce no pathological changes. Normal handling precautions should be exercised with this chemical until long-term, chronic toxicity evaluation has been terminated, and current confirmatory experiments have been completed.

CONCLUSIONS

Three years of laboratory and field testing have confirmed the diverse insecticidal properties of Sevin. In several geographic locations, it has been reported to be effective against resistant strains of codling moth and pink bollworm. No instances of incompatibility with other pesticides (glyodin, DDT, ferbam, captan, maneb, etc.) have been encountered. Laboratory tests performed with normal and high rates of wettable powder or dust formulations have not produced plant injury on important crop plants such as bean, corn, tomato or cotton. The residual life of this new insecticide is of intermediate duration, occupying a position about midway between that of relatively short-lived TEPP and that of very persistent DDT. Cumulative mammalian toxicological findings indicate that this is an insecticide of relatively low toxicity hazard.

⁶ Personal communication on work in progress from: C. P. Carpenter, C. S. Weil and H. F. Smyth, Jr., Mellon Institute, Pittsburgh, Pa., 1956.

Experiments designed to reflect systemic activity have indicated that small quantities of Sevin are translocated in plant tissue. Control of those insects which are most susceptible to this chemical (example, Mexican bean beetle) can be affected by this type of application. However, completely negative results against sucking insects (bean aphid and red spider mite) with soil incorporation indicate that possible commercialization of the systemic properties of this chemical would be of doubtful advantage. Preliminary results of seed treatment experiments appear more promising as the insecticidal properties of Sevin can be demonstrated in isolated bean leaves of plants grown from treated seed. These have been found to be toxic to the southern armyworm as well as the Mexican bean beetle.

On the strength of the insecticidal properties and physico-chemical characteristics described for 1-naphthyl *N*-methylcarbamate, expanded development at the experiment station level will be continued for the ensuing season.

ACKNOWLEDGMENTS

The authors acknowledge with thanks the assistance of Drs. Richard C. Back and Philip Granett who were instrumental in compiling the field data. Laboratory evaluation was obtained by Messrs. Anthony A. Sousa and Andrew J. Borash.

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